THE ROLE OF UBIQUITIN CONJUGATING ENZYMES IN *DROSOPHILA* DEVELOPMENT

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A thesis presented for the degree of PhD

University of Edinburgh

1996

ACKNOWLEDGEMENTS:

I would like to thank past and present members of the Finnegan group for their help, encouragement and friendship, namely David himself, Angela, Apinunt, Eve, Hong mei, Ivan, Jill, Julie, Laura, Maki, Petra (without whom none of this would have been possible...) and Susan. Special thanks to "honorary" Finnegan group member Jeff Bond for advice and squash matches!

Many thanks go to other people who have helped me with particular parts of my work, especially Frank Wright for explaining dendrograms, Michelle Cooper who did a lot of the yeast work for me, Andrew Jarman for discussions about GAL4 experiments and allowing me to use his microsope, and Helena Thomaides who worked as a Summer student on the ubiquitin project.

The students who worked under my supervision, Will Sauer and Julie Beattie, also deserve a mention, not only for the pleasure I had in teaching them (I hope I didn't push you two too hard!), but also for their work and company in the lab..

I am indebted to other members of I.C.M.B.:- Adrian, Gareth, Hari, Peter (of course!), Sarah, and Simon. If these people hadn't been around, life would have been a lot less enjoyable. Thank you also to many past and present 6th floor Darwin people, especially Vicki, Laurie and Jules. Friends I would like to mention outside of work are Ed, Barbara, Martin, Colin and Evelyn, Taza and Lorraine.

Finally I thank my family for their love and understanding.

ABBREVIATIONS:

A	- adenosine
Å	- Ångström
amp	- ampicillin
AP	- alkaline phosphatase
ATP	- adenosine 5'-triphosphate
βgal	- β-galactosidase
bp	- base pair
BPB	- bromophenol blue
BSA	- bovine serum albumin
C	- cytidine
°C	- degrees celsius
cDNA	- complementary DNA
Ci	- curie
cpm	- counts per minute
d	- deoxy
DAB	- 3,3'-diaminobenzidine
dd	- dideoxy
DEPC	- Diethyl pyrocarbonate
DIG	- digoxygenin
DMSO	- dimethyl sulphoxide
DNase	- deoxyribonuclease
dNTP	- deoxyribonucleoside triphosphate
DTT	- dithiothreitol
EDTA	- diaminoethanetetra-acetic acid
g	- gram
G	- guanosine
GST	- glutathione S-transferase
hr	- hour
HEPES	- N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid]
HRP	- horse radish peroxidase
IPTG	- isopropyl-β-D-thiogalactopyranoside
kb(s)	- kilobase(s)
kDa	- kiloDaltons

l	- litre
L-broth	- Luria broth
M	- molar
Mb(s)	- megabase(s)
mg	- milligram
min	- minute
ml	- millilitre
mol	- mole
MOPS	- 3-[N-Morpholino]propanesulphonic acid
MW	- molecular weight
NBT	- 4-Nitro blue tetrazolium chloride
ng	- nanogram
nm	- nanometre
O.D.	- optical density
OLB	- oligo-labelling buffer
OrR	- OregonR
p	- plasmid
p	- pico
PAGE	- polyacrylamide gel electrophoresis
PBS	- phosphate buffered saline
PCR	- polymerase chain reaction
p.d.	- potential difference
PEG	- polyethylene glycol
PIPES	- piperazine-N,N'-bis [2-ethane-sulphonic acid]; 1,4-piperazine
POD	- peroxidase
PVDF	- polyvinylidene fluoride
RNase	- ribonuclease
rpm	- revolutions per minute
sec	- second
SDS	- sodium dodecyl sulphate
ssDNA	- single-stranded deoxyribonucleic acid
T	- thymidine
TEMED	- N,N,N'-tetramethylethylenediamine
T_m	- melting temperature

Tris	- tris (hydroxymethyl) aminomethane
U	- uridine
UAS	- upstream activator sequence
µg	- microgram
µl	- microlitre
UV	- ultraviolet
v	- volt
v	- volume
v/v	- volume per volume
w/v	- weight per volume
w-t	- wild type
X-gal	- 5-bromo-4-chloro-3-indol-β-D-galactopyranoside
X-phosphate	- 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt

AMINO ACIDS

Amino acids	Three letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABSTRACT:

The ubiquitin system forms the major pathway for selective degradation of abnormal, damaged and short-lived proteins in eukaryotes. Ubiquitin becomes attached to substrate proteins to target them for degradation via three types of enzyme: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin protein ligases (E3). E1 enzymes are only necessary to activate ubiquitin and transfer it to an E2 enyzme. Many E2s are present within a eukaryote, and some can ubiquitinate substrates without the aid of an E3 enzyme. Studies in *Saccharomyces cerevisiae* have shown particular E2s function in processes as diverse as DNA repair, peroxisome biogenesis and cell cycle control. Much of the substrate specificity for ubiquitination is thought to reside within ubiquitin conjugating enzymes.

Control of protein levels by selective degradation is important for cell differentiation and developmental processes. To allow specific spatial and temporal distribution of proteins which are key regulators of development, highly selective degradation is required. Ubiquitin-dependent proteolysis also appears essential for developmentally programmed cell death. The experiments in this thesis describe attempts to address the role of the ubiquitin system in development using *Drosophila melanogaster* as a model system. As ubiquitin conjugating enzymes are thought to each ubiquitinate a subset of proteins, these were the enzymes investigated.

Four E2 enzyme genes had previously been cloned in *Drosophila*, and a further E2 was identified by a polymerase chain reaction screen. The gene was cloned, sequenced and named *UbcD4* (ubiquitin conjugating enyzme in *Drosophila* number 4). *UbcD4* encodes a protein homologous to bovine $E2_{25K}$ and yeast UBC1. All five E2 genes were investigated for developmentally regulated transcription. High levels of E2 mRNA were observed in adult females, early embryos and larvae, suggesting specific developmental roles for the ubiquitin system at these stages. Ubiquitination may have a major function in the developing central nervous system, as RNA from all known E2s was found to be abundant in this tissue in embryos.

Ubiquitin conjugating enzymes become linked to ubiquitin via a thiol ester bond to an active site cysteine residue within the E2. As a first step to determining the function of E2s in *Drosophila*, active site mutants of the *Drosophila* E2 DHR6 were overexpressed. This resulted in lethality, probably at the late pupal stage of development. *Dhr6* is the

homologue of the *Saccharomyces cerevisiae RAD6* gene, which is essential for various DNA repair functions. Results suggest *Dhr6* is also an essential gene.

The implications of these results for the roles of E2s in development are discussed.

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CHAPTER 1

INTRODUCTION

1.1 PROTEIN DEGRADATION

1.1.1 WHY IS PROTEIN DEGRADATION NECESSARY?

Although DNA stores the genetic information necessary to make an organism, most of this information is translated into proteins. It is proteins that determine the shape and structure of cells, and proteins are the main mediators of molecular recognition and catalysis.

Many different types of protein exist in an organism, ranging from structural proteins and "housekeeping" enzymes necessary for vital functions in all cells, to cell-specific regulatory proteins. Some of these proteins are practically stable molecules, whilst others have half-lives of a few minutes. The half-life of a protein is determined by highly selective intracellular protein degradation. Most structural proteins are relatively stable, whereas regulatory proteins have concentrations which change promptly with alterations in the state of the cell. These proteins must have short half-lives so that their levels can be rapidly changed in response to appropriate stimuli.

Cells do not always produce components of protein complexes in precise amounts, and must selectively degrade any protein component left unassembled (figure 1.1). A system to identify and destroy abnormally assembled proteins is required. Denatured, damaged or misfolded proteins, and those containing oxidized or other abnormal amino acids, must also be recognised and degraded. The proteolytic system must be able to distinguish complete proteins in abnormal conformations, from nascent polypeptides on ribosomes.

The ubiquitin system is the major pathway for selective protein degradation within eukaryotic cells (Ciechanover *et al.*, 1984), performing all the above functions. However, important degradation pathways also operate in the endoplasmic reticulum (E.R.) and lysosomes in eukaryotic cells, and most prokaryotes do not have ubiquitin.

1.1.2 THE UBIQUITIN PATHWAY:

A schematic diagram of the ubiquitin pathway (reviews: Finley and Chau, 1991; Jentsch, 1992; Ciechanover, 1994; Deshaies, 1995a.) is shown in figure 1.2. Ubiquitin becomes attached to target proteins via three types of enzyme. First the carboxyl terminus of ubiquitin is activated in an ATP-dependent reaction. This

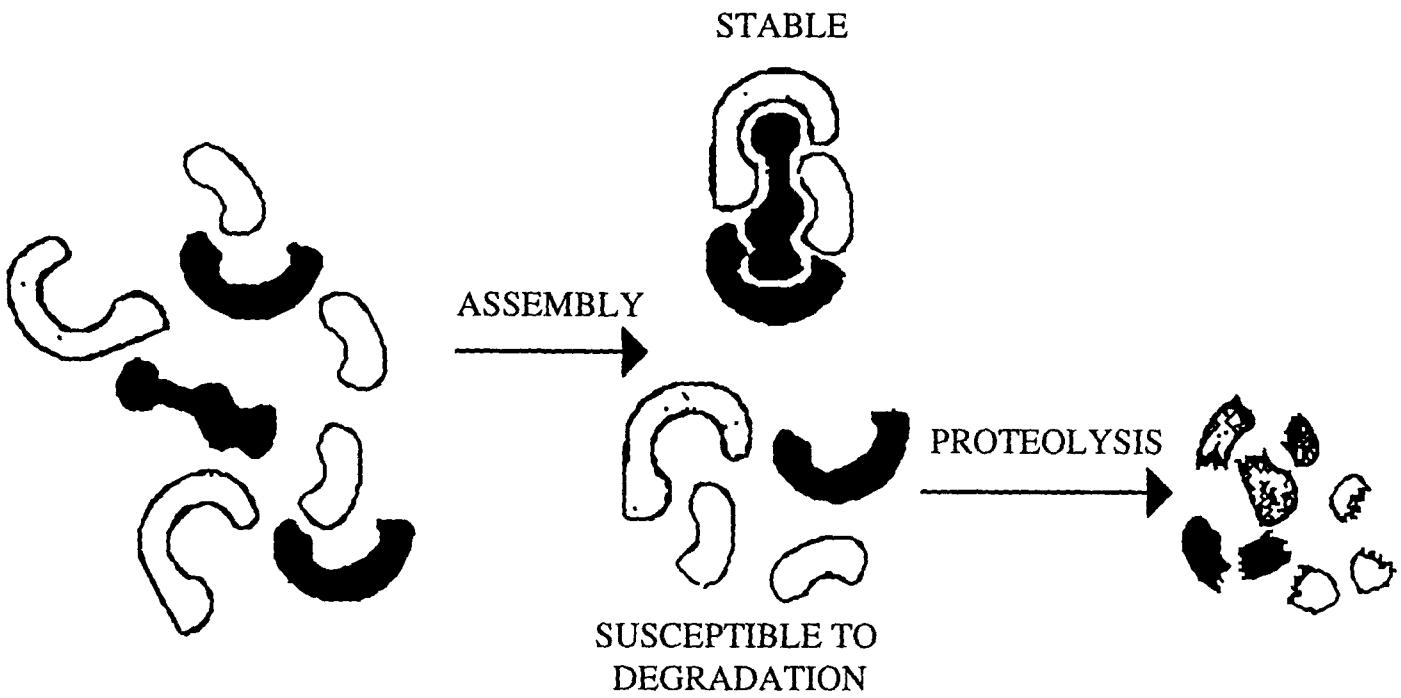


Fig. 1.1 Extra components of protein complexes are degraded to prevent accumulation in the cell (after Alberts *et al.*, 1994).

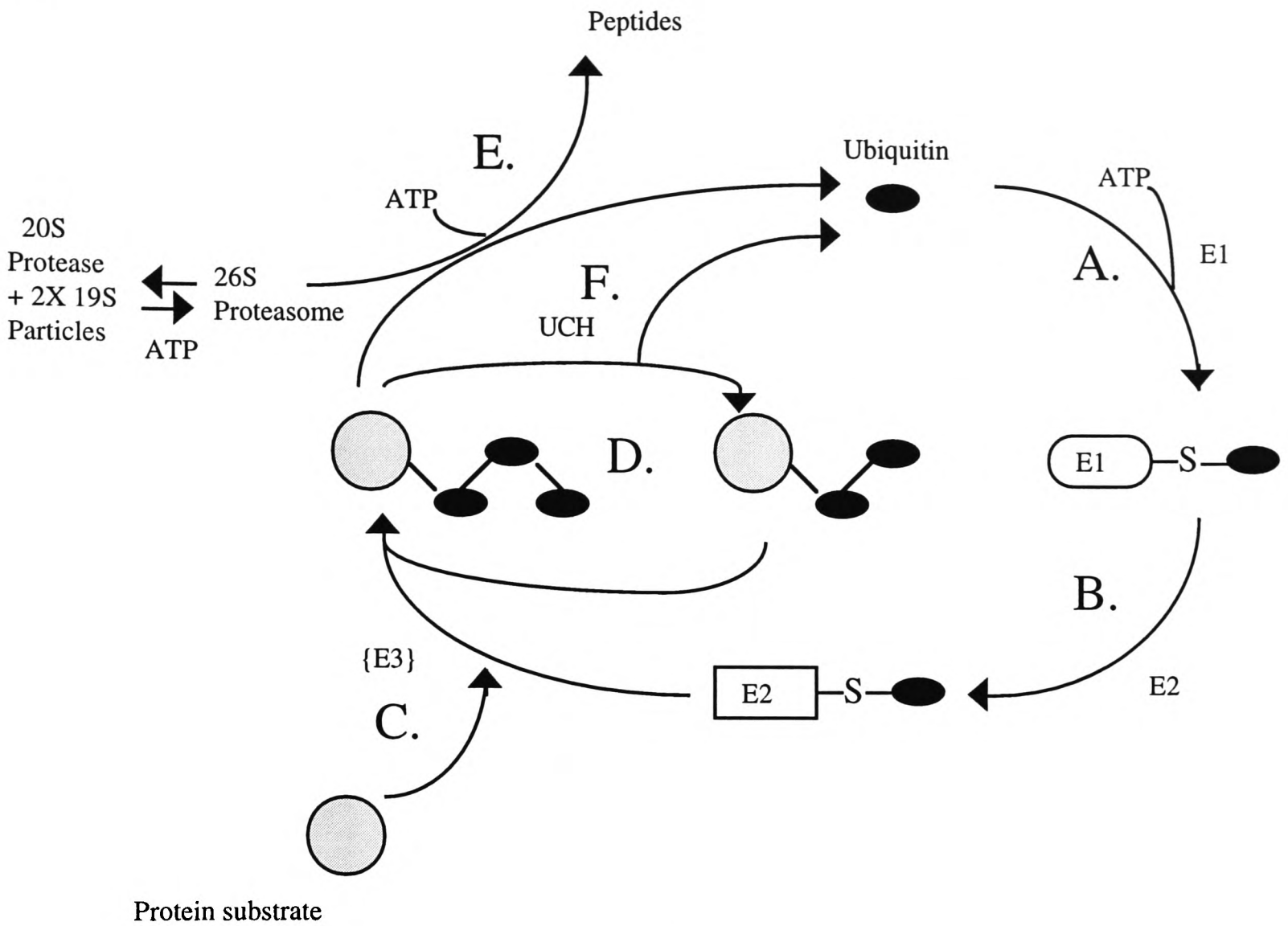


Fig. 1.2 The Ubiquitin Pathway (after Hochstrasser, 1992).

A. Activation of ubiquitin via an E1 enzyme.
B. Transfer of ubiqutin to a ubiquitin conjugating enzyme (E2).
C. Conjugation of ubiquitin to the protein substrate from an E2, sometimes requiring an E3 ubiquitin protein ligase.
D. Multiubiquitination of the protein substrate by repeating A., B., and C., and addition of ubiquitin moieties to Lys48 of the previous ubiquitin.
E. Cleavage of the multiubiquitinated protein to peptides by the 26S proteasome.
F. Removal of ubiquitin chains, and cleavage of the chains by ubiquitin carboxyl-terminal hydrolases (UCH) to ubiquitin monomers which are recycled in the pathway.

involves formation of a ubiquitin adenylate, followed by transfer of the C-terminus to a thiol group on the active site Cys residue of a ubiquitin activating enzyme, or E1, which catalyzes the activation reaction (Haas and Rose, 1982; Haas *et al.*, 1982). Ubiquitin is then transferred to one of several ubiquitin conjugating enzymes, or E2s, to generate a similar thiol-ester linkage between ubiquitin and an active site Cys residue in the E2 (Pickart and Rose, 1985). The E1 enzyme probably binds to the N-terminus of the E2 during the transfer of ubiquitin (Cook *et al.*, 1992). E2s donate ubiquitin to an acceptor Lys of the target protein (Chau *et al.*, 1989), sometimes with the aid of a ubiquitin-protein ligase or E3 for substrate recognition (Ciechanover and Schwartz, 1989). E3s may also bind ubiquitin via a thiol-ester linkage (see 1.8). The process can then be repeated, and the first ubiquitin is ubiquitinated on Lys48 (Chau *et al.*, 1989). Multiubiquitination of the substrate protein, via Lys48 isopeptide linkage, is sufficient for degradation (Gregori *et al.*, 1990) and greatly enhances the process (Chau *et al.*, 1989).

The multiubiquitinated protein is degraded via the 26S proteasome complex (see 1.9). Ubiquitin itself is a stable protein, and is recycled in the pathway after cleavage from the chains by ubiquitin C-terminal hydrolases (UCHs: see 1.10).

1.1.3 PROKARYOTIC PROTEASES:

Although some eubacteria and archaebacteria have been found to contain ubiquitin and proteasomes (see 1.2.1), most prokaryotes degrade proteins via different classes of ATP-dependent proteases (review: Gottesman and Maurizi, 1992). *Lon*-like proteases are multimers of identical polypeptide chains, each with a single proteolytic site, and an ATPase site. *Clp*-like proteases have a single type of proteolytic subunit, *ClpP*, associated with one or more ATPase hydrolysing units, *ClpA*. This forms a high molecular weight multimer (Maurizi, 1994), thought to be similar to eukaryotic 26S proteasomes.

1.1.4 EUKARYOTIC NON-UBIQUITIN PROTEIN DEGRADATION:

1.1.4.1. Protein degradation in the endoplasmic reticulum (E.R.):

Membrane proteins and lysosomal hydrolases are synthesised in the rough E.R. For exit of a protein from the E.R., it must be correctly folded and assembled.

Abnormally folded or assembled proteins are retained in the E.R., either bound to the special binding protein (BiP), or in aggregates, and are eventually degraded.

The signal for degradation of proteins in the E.R. is exposure of internal domains, usually buried when the protein has adopted the correct conformation. These are exposed in assembly intermediates, but are also the sites for BiP binding (Schmitz *et al.*, 1995). BiP suppresses degradation of normal protein assembly intermediates by masking the internal domains, which are signals both for BiP binding and degradation.

E.R. protein degradation was thought to be separate from ubiquitin-dependent protein degradation, which was mainly thought to occur in the cytoplasm. However, the *Saccharomyces cerevisiae* ubiquitin conjugating enzyme (E2) UBC6 (see 1.7.1.5) localises to the E.R., and appears to mediate selective degradation of E.R. membrane proteins (Sommer and Jentsch, 1993). This suggests a role for the ubiquitin system in E.R. protein degradation.

1.1.4.2 Lysosomal protein degradation:

Lysosomes contain acid hydrolase proteases. Proteins to be digested can come from the external medium via endocytosis, autophagy of organelles, or specific import of cytosolic proteins bearing KFERQ signals on their surfaces (Terlecky and Dice, 1993).

The ubiquitin pathway was previously thought to be separate from lysosomal degradation (figure 1.3). Ubiquitination has now been shown to be involved in receptor mediated endocytosis (review: Hurtley, 1996; see 1.12.3) and the proteolytic events responsible for transformation of autolysosomes to residual bodies (Leck *et al.*, 1992). This implies that lysosomal-mediated protein degradation often requires protein ubiquitination.

Ubiquitination may be a universal signal for protein degradation in eukaryotes.

1.2 THE UBIQUITIN PROTEIN:

Ubiquitin is an abundant, 76 amino acid protein, first isolated from bovine thymus (Goldstein *et al.*, 1975), and found in all eukaryotic cells screened. It has one of the most conserved sequences known; 72 amino acids are invariant throughout

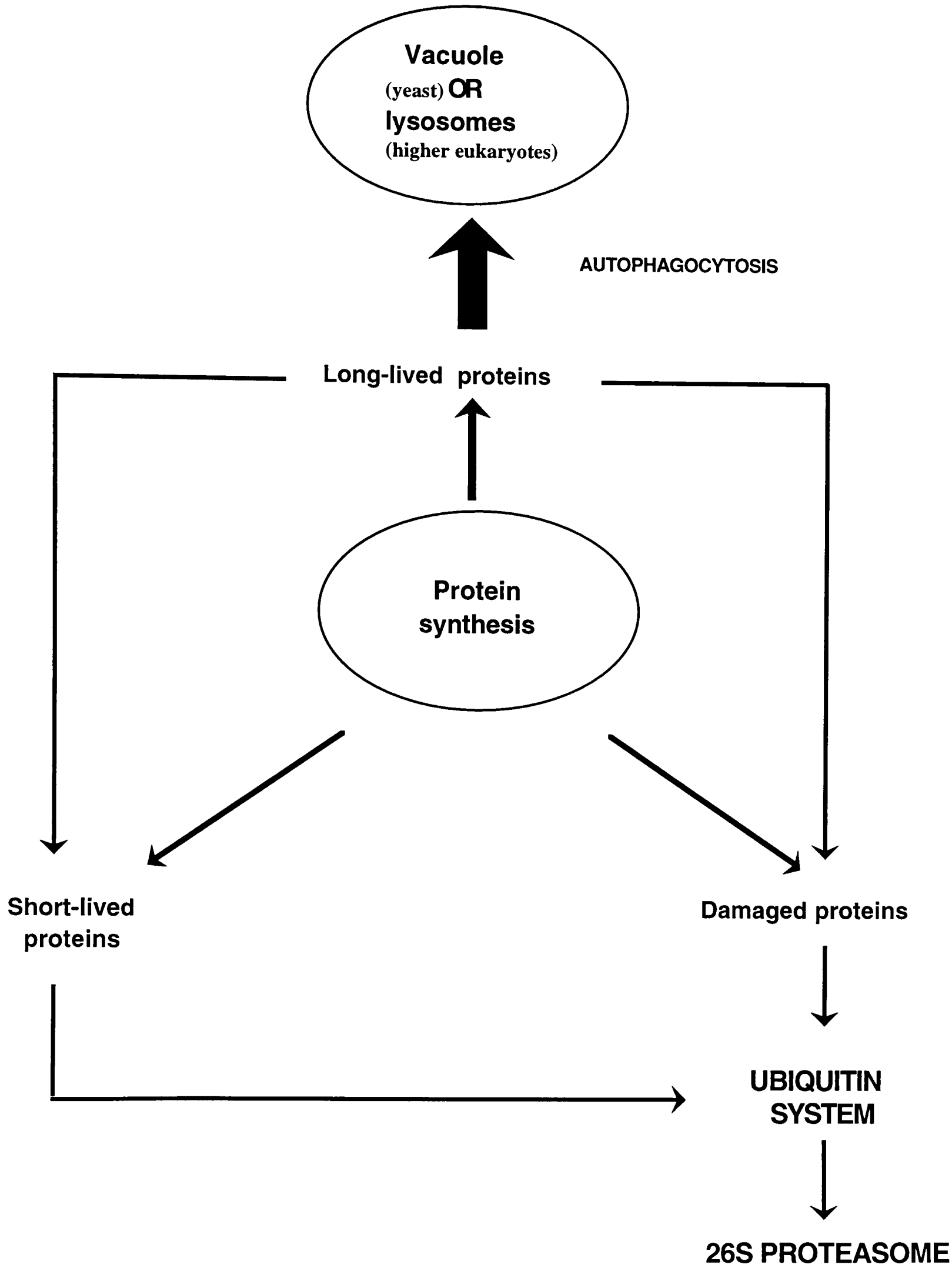


Fig. 1.3 Protein degradation via the ubiquitin pathway, and lysosomes.
These two systems were thought to be separate, but ubiquitin has now been shown to be involved in some lysosomal protein degradation. Also degradation of long-lived proteins may require the proteasome, if not other parts of the ubiquitin pathway.

fungi, plants and vertebrates (Foster *et al.*, 1990). Ubiquitin is a highly compact globular protein (figure 1.4) with three faces, acidic, basic and hydrophobic. Only the four C-terminal residues, the site of ubiquitin-protein conjugation, protrude from the core (Vijay-Kumar *et al.*, 1987).

The strict evolutionary conservation of ubiquitin suggests each residue has an essential role in folding, stability, or function. The C-terminal Gly forms an isopeptide bond with the ε-amino group of a specific Lys residue in the target protein (Chau *et al.*, 1989). When Gly is changed to Ala at this position in *Saccharomyces cerevisiae*, ubiquitin becomes irreversibly bound to proteins, and phenotypes expected for ubiquitin-deficiency, such as slow growth and sensitivity to environmental stress, are seen (Ecker *et al.*, 1987; Hodgins *et al.*, 1992). Polyubiquitin chains form when further ubiquitin monomers become covalently linked, by their C-terminal Gly residues, which are again essential (Hodgins *et al.*, 1992), to Lys48 of the previous monomers (Chau *et al.*, 1989). If Lys48 is altered, it acts as an inhibitor of ubiquitin dependent protein degradation (Bachmair *et al.*, 1990). Three Arg residues (42, 72, 74) are necessary for interactions with E1 (Burch and Haas, 1994), but most specific residues appear necessary just to produce the tight globular structure.

1.2.1 UBIQUITIN IN VIRUSES AND PROKARYOTES:

The ubiquitin system is not wholly confined to eukaryotes. Ubiquitin conjugating enzymes have been found in viruses (e.g. Hingamp *et al.*, 1992) probably as a means for exploiting host regulatory mechanisms. The ubiquitin protein is present in eubacteria (Durner and Boger, 1995) and archaebacteria (Wolf *et al.*, 1993). A proteasome, architecturally indistinguishable from that of eukaryotes, but simpler in subunit composition (see 1.9), is also present in archaebacteria (Dahlmann *et al.*, 1989), and a 20S eubacterial proteasome was recently discovered (Tamura *et al.*, 1995).

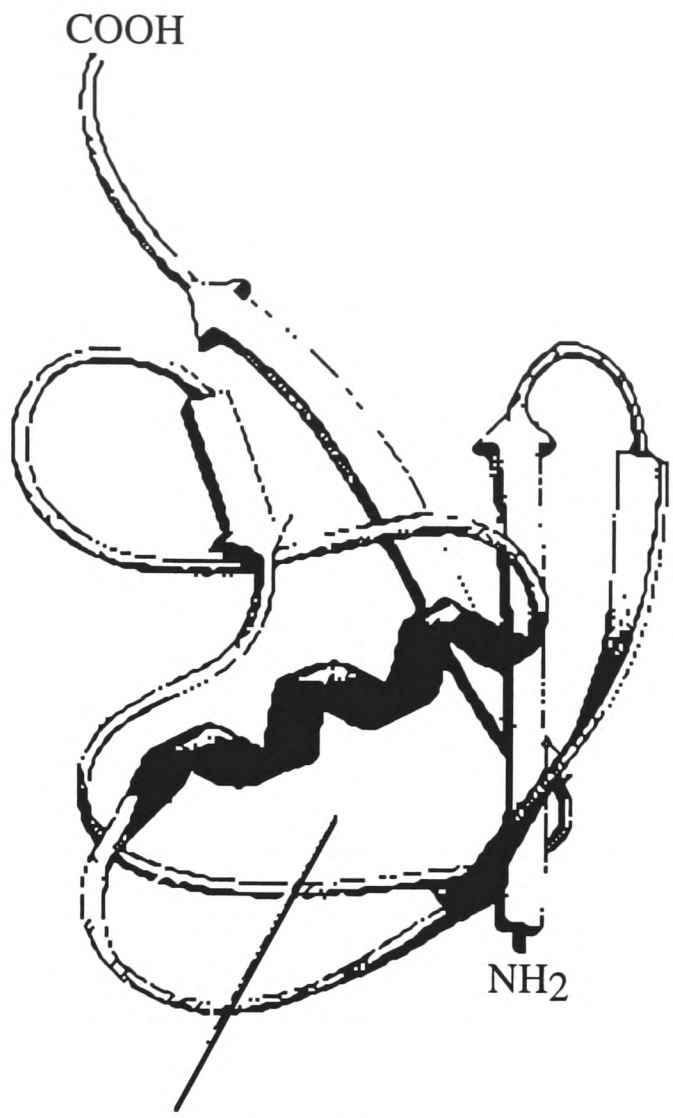


Fig. 1.4 The three-dimensional structure of ubiquitin (after Vijay-Kumar *et al.*, 1985).

1.3 CONTROL OF FUNCTION OF THE UBIQUITIIN PATHWAY:

1.3.1 POLYUBIQUITIN CHAINS:

The Lys48-linked polyubiquitin chains play a crucial role in protein degradation *in vivo* (Finley *et al.*, 1994). The structure of tetraubiquitin shows these chains can be formed as repeating diubiquitin subunits, with multiple hydrophobic contacts amongst all four ubiquitins (Cook *et al.*, 1994). However, polyubiquitin chains may not always be linked via Lys48, as these chains were not necessary for the survival of stress in *Saccharomyces cerevisiae* (Finley *et al.*, 1994). Instead Lys63-linked chains appeared to play an important role (Arnason and Ellison, 1994). Under stress conditions, Lys29-linked chains may also be present, and Lys29, but not Lys48, was found to be necessary for degradation of an artificially made short-lived protein (Johnson *et al.*, 1995). Structurally different multiubiquitin chains may have distinct functions in ubiquitin-dependent protein degradation. However, most of this work involved mutating the Lys residues of ubiquitin, and observing the effects of degradation of a specific protein under different conditions. It is not known if these different chains do actually exist in these experiments, and if they do, they may be artifacts due to the Lys48 residue not being present for attachment of further ubiquitin monomers, and a different Lys residue having to be used. Gregori *et al.* (1990) mutated all the Lys residues of ubiquitin separately and found only Lys48 was essential.

Homopolymeric ubiquitin chains of 8-12 ubiquitin monomers, conjugated to a substrate Lys, result in ten times faster degradation than monoubiquitin (Chau *et al.*, 1989). Ubiquitin residues Lys8, Ile44 and Val70 do not affect attachment of ubiquitin to substrates, but are necessary for degradation of the resulting conjugates (Beal *et al.*, 1996). The human proteasome regulatory subunit S5a binds Lys48-linked multiubiquitin chains, of four or more ubiquitins, with increasing affinity as a function of chain length (Deveraux *et al.*, 1994), and the ubiquitin residues mentioned above are necessary for this binding (Beal *et al.*, 1996). The amino acids Lys8, Ile44 and Val70 form repeating patches on the surface of multiubiquitin chains, implying hydrophobic interactions between the patches and S5a contribute to the enhanced proteolytic targeting of multiubiquitin chains. An *Arabidopsis* homologue of S5a has been found (Vannocker *et al.*, 1996), and similar proteins are also present in yeast, *C.elegans* and rice. This protein is an essential ubiquitin recognition component of the 26S proteasome.

1.3.2 MONOUBIQUITINATION:

Not all proteins that become monoubiquitinated proceed to be polyubiquitinated and degraded. Some proteins remain stable as monoubiquitinated forms. The first protein found conjugated to ubiquitin was histone H2A (uH2A; Goldknopf and Busch, 1977). Fifteen percent of H2A and two percent of H2B are monoubiquitinated in mammalian cells, and the degree of ubiquitination varies with the cell cycle (Raboy *et al.*, 1986), decreasing in metaphase cells. In slime mould, cleavage of ubiquitin from uH2A and uH2B is a very late event in chromosome condensation to metaphase chromosomes, and ubiquitination is an early event in their decondensation (Mueller *et al.*, 1985).

Monoubiquitination may modulate the activity of proteins, rather than labelling them for degradation. Other proteins shown to be stably monoubiquitinated include calmodulin (Parag *et al.*, 1993) and actin in *Drosophila* flight muscles (Ball et al., 1987). The function of these monoubiquitinations is unknown.

1.3.3 PHOSPHORYLATION CONTROL:

Protein ubiquitination is regulated by phosphorylation. Phosphorylation may make a protein susceptible to degradation; phosphorylation of Ser residues in IκBα targets the protein to the ubiquitin-proteasome pathway (Chen *et al.*, 1995; see 1.11.1). Alternatively, ubiquitin pathway enzymes can be activated by phosphorylation: a rabbit reticulocyte E1 increases in enzymic activity on phosphorylation (Kong and Chock, 1992). A HeLa cell E2, $E2_{32K}$, when phosphorylated leads to an increase in ubiquitination of H2A (Kong and Chock, 1994) which may in turn play a role in cell cycle control. Clam cyclin specific E3-C is a ubiquitin protein ligase found as a complex known as the cyclosome, which is necessary for degradation of cyclins at the end of metaphase (see 1.11.4). The E3 activity of the cyclosome is activated by cdc kinase, and inactivated by phosphatase (Lahavbaratz *et al.*, 1995). Phosphorylation control has not been fully investigated, but may be a general means of activating ubiquitin-dependent degradation pathways.

1.4 UBIQUITIN-ENCODING GENES:

Ubiquitin is encoded by a gene family whose primary translation products are fusion proteins. Ubiquitin genes code for an amino terminal ubiquitin fused to a large or small ribosomal subunit protein, or for head-to-tail polyubiquitin fusion proteins

with no spacer regions between ubiquitin moieties. Ubiquitin is released, for conjugation to proteins, by C-terminal hydrolases (see 1.10) which cleave C-terminal of ubiquitin moieties (Özkaynak *et al.*, 1984). The C-terminal ubiquitin moiety of polyubiquitin gene products has at least one additional amino acid at the C-terminus (Özkaynak *et al.*, 1984) to prevent unprocessed polyubiquitin proteins being conjugated to substrates.

Arabidopsis thaliana has 14 ubiquitin-encoding genes:- 5 polyubiquitin genes, 4 ubiquitin-ribosomal protein genes, and 5 ubiquitin-like genes, thought to be pseudogenes (Callis *et al.*, 1995). Humans also have three types of ubiquitin-encoding genes (Wiborg *et al.*, 1985; Cowland *et al.*, 1988):- ubiquitin-ribosomal fusions, polyubiquitin genes with 9 repeats, and polyubiquitin genes with 3-4 repeats of ubiquitin. The tetrameric gene is a pseudogene, possibly created by unequal crossing-over of ancestral trimeric genes. Multiple polyubiquitin genes in eukaryotes probably arose from gene duplication and unequal crossing-over events.

1.4.1 *S.CEREVISIAE* UBIQUITIN-ENCODING GENES:

A family of four ubiquitin-encoding loci are present in yeast (Özkaynak *et al.*, 1984; Özkaynak *et al.*, 1987). *UBI1*, *2* and *3* are ubiquitin monomer genes (Özkaynak *et al.*, 1987) fused to ribosomal protein encoding regions (Finley *et al.*, 1989). *UBI1* and *2* encode the same essential large ribosomal subunit protein of 52 amino acids, and *UBI3* encodes an essential small ribosomal subunit protein of 76 amino acids (Finley *et al.*, 1989), as well as ubiquitin. The ubiquitin moiety of the ribosomal fusion proteins facilitates ribosome assembly, and may act as a molecular chaperone (Finley *et al.*, 1989; Finley and Chau, 1991). Fusion of ubiquitin to ribosomal proteins allows co-regulation of the ubiquitin system with the translational activity of the cell (Redman and Rechsteiner, 1989).

Other eukaryotes have the same ubiquitin fusion proteins as those in yeast. The larger C-terminal extension protein (CEP) in rats is 40S ribosomal subunit protein S27a (Redman and Rechsteiner, 1989), and human *HUbCEP80* can complement yeast *ubi3* mutants. Complementation does not occur when only CEP80 is expressed (Monia *et al.*, 1990), again suggesting ubiquitin increases the translational efficiency or stability of the cotranslated ribosomal proteins.

The yeast polyubiquitin gene, *UBI4*, has five head-to-tail ubiquitin-coding repeats (Özkaynak *et al.*, 1987). It is strongly induced in stress conditions, such as starvation and high temperature, to satisfy the increased ubiquitin requirement

(Özkaynak *et al.*, 1987), and is an essential component of the stress response (Finley *et al.*, 1987).

1.4.2. *DROSOPHILA* UBIQUITIN-ENCODING GENES:

The ubiquitin-encoding genes of *Drosophila melanogaster* are shown in figure 1.5. Again a multigene family is present.

The polyubiquitin gene, *Ubip*, was isolated from an early ecdysone puff at 63F (Izquierdo *et al.*, 1984). Expression of the gene is constant throughout development for a particular strain of *Drosophila melanogaster* (Arribas *et al.*, 1986). The gene is polymorphic among different stocks and strains, containing either 15 or 18 tandem repeats depending on the stock analysed (Lee *et al.*, 1988). The C-terminal ubiquitin repeat of the polyubiquitin fusion protein has three extra amino acids:-Ala, Gln, and Ile. Expression of the polyubiquitin gene is not tissue specific, but is induced under heat shock conditions (Lee *et al.*, 1988).

Two *Drosophila* ubiquitin monomer genes have been cloned and characterised. *DUb52* (Cabrera y Poch *et al.*, 1990) has a 52 amino acid extension protein, found to be a 60S ribosomal subunit protein (Redman *et al.*, 1994). *DUb52* is expressed constitutively throughout development and is particulary abundant in ovaries (Cabrera *et al.*, 1992). The *Drosophila* homologue of *UBI3, DUb80*, encodes a ubiquitin monomer fused to the equivalent 40S ribosomal protein of *UBI3* (Lee *et al.*, 1988; Barrio *et al.*, 1994). It has a constitutively expressed 0.9kb mRNA, particularly abundant under conditions of high protein synthesis, such as in ovaries and exponentially growing cells (Barrio *et al.*, 1994), but is not inducible by heat shock (Lee *et al.*, 1988).

Izquierdo (1994) reported another polyubiquitin gene on the X chromosome of CantonS *Drosophila melanogaster*. In polytene chromosomes, ubiquitin was found to be mainly associated with the compact and stabilised DNA of bands, rather than the more decondensed DNA of puffs and interbands (Izquierdo, 1994) suggesting that ubiquitination may stabilise histones. The pattern of immunostaining with ubiquitin matched the banding pattern of polytene chromosomes, but there was also weaker staining of interbands and puffs. The bands may stain more strongly due to the greater concentrations of DNA and therefore higher amounts of histones present than in other less condensed chromosomal regions.

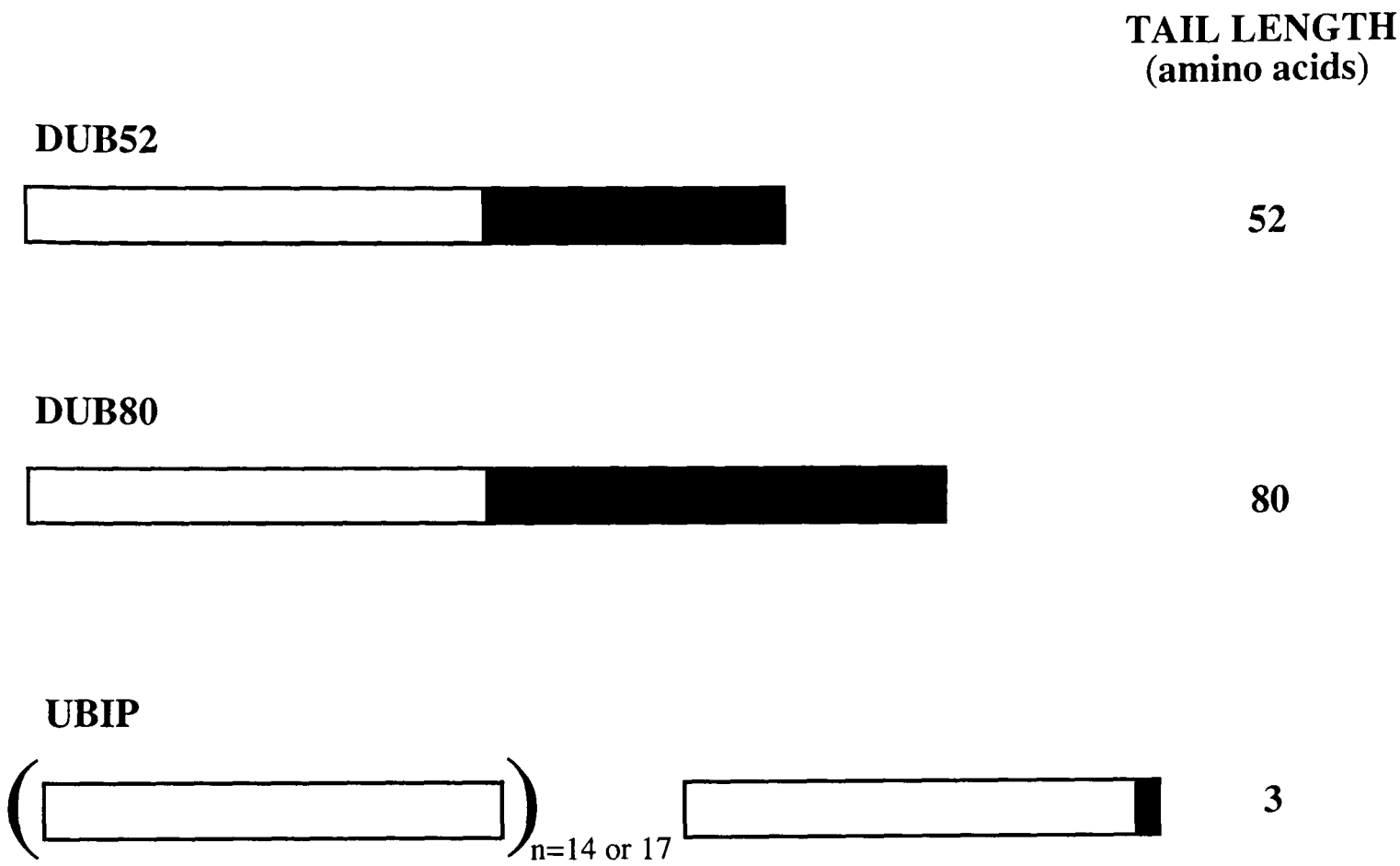


Fig. 1.5 The proteins encoded by *Drosophila* ubiquitin genes.

= Ubiquitin = C-terminal fusion

1.5 SUBSTRATE SELECTION:

The method of substrate selection for ubiquitination is still unsolved (reviews: Ciechanover and Schwartz, 1989; Rechsteiner, 1991; Hershko and Ciechanover, 1992; Ciechanover, 1994). This section describes various models, and signals that appear to be important.

1.5.1. THE N-END RULE: A BIPARTITE SIGNAL:

The *in vivo* half-life of a protein can be determined by the amino acid residue at the N-terminal position: the N-end rule (review: Varshavsky, 1992). Measurement of the *in vivo* half-lives of 20 different species of β-galactosidase proteins in *S.cerevisiae* revealed they vary from more than 20hr to less than 3 min (table 1.1), depending on the N-terminal amino acid, which was then classified as "stabilising" or "destabilising" (Bachmair *et al.*, 1986). This reflects the affinity of an E3 protein, UBR1 or N-recognin in *S.cerevisiae*, for the N-terminal residues of proteolytic substrates (Bartel *et al.*, 1990).

UBR1 selects N-end rule substrates by binding to primary destabilising N-terminal residues (table 1.1). It has at least two substrate binding sites, Type I specific for basic residues Arg, Lys, and His, Type II specific for bulky hydrophobic residues Phe, Leu, Trp, and Ile (Reiss *et al.*, 1988; Gonda *et al.*, 1989). Tertiary destabilising N-terminal residues Asn and Gln are so named because they are first converted by N-terminal amidase, encoded by the *NTA1* gene (Baker and Varshavsky, 1995) to secondary destabilising residues Asp and Glu. These in turn are conjugated to Arg, a primary destabilising residue, by Arg-tRNA-protein transferase (the *ATE1* gene product; Ferder and Ciechanover, 1987) to allow recognition by UBR1.

The degradation signal does not consist solely of the N-terminal amino acid. A second determinant, a specific Lys which becomes polyubiquitinated, is also essential (Bachmair and Varshavsky, 1989). The sequences surrounding the Lys are not important, and the two determinants can be located on different subunits of a multisubunit protein, and still target the protein for degradation via "trans recognition" (Johnson *et al.*, 1990), provided they are in spatial proximity. Only the subunit containing the available Lys is proteolysed. The site for recognition of the Lys may be on an E2 as UBC2 functions with UBR1 in the N-end rule pathway. UBC2 is physically associated with UBR1 via its 23 acidic C-terminal amino acids (Madura *et al.*, 1993; see 1.7.1.2).

N-TERMINAL RESIDUE	HALF-LIFE	
Met Pro Val Gly Thr Ser Ala Cys	>20h	STABILISING
Glu Ile	~30min	DESTABILISING
Gln His Tyr	~10min	
Asn Asp Trp Leu Phe Lys	~3min	
Arg	~2min	

Table 1.1 Half life of β-galactosidase with different N-terminal amino acids in *Saccharomyces cerevisiae* (after Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989; Gonda *et al.*, 1989).

The cellular importance of the N-end rule pathway is unclear, as *ubr1* mutants, which lack this proteolytic route, are almost phenotypically normal (Bartel *et al.*, 1990). The majority of cellular proteins are acetylated at their N-termini, so are blocked for N-end rule degradation. Degradation of N-acetylated proteins is E1-dependent (Mayer *et al.*, 1989), so they must be recognised for ubiquitin-dependent degradation via a different signal.

1.5.2 SPECIFIC AMINO ACID SEQUENCES AS DEGRADATION SIGNALS:

1.5.2.1 PEST sequences:

Rogers *et al.* (1986) noticed that short-lived proteins have stretches of amino acids enriched in Pro, Glu, Ser, and Thr (PEST regions), flanked by clusters of positively charged amino acids. Thirty out of thirty-two short-lived proteins with known sequences were found to contain one or more PEST sequences (Rechsteiner, 1990). PEST sequences appear to be signals for rapid degradation, but neither the mechanism of how the signal targets for degradation, nor the system recognising the signal has been identified.

1.5.2.2. *Deg1* and *Deg2*:

The short-lived MATα2 repressor is degraded by the ubiquitin system (Hochstrasser *et al.*, 1991; see 1.7.1.6 and 1.11.1). It has 2 degradation signals, one requiring residues 53-67 in the N-terminal region (*Deg1*), the other residing in residues 136-140 in the C-terminal domain (*Deg2*; Hochstrasser and Varshavsky, 1990). *Deg1* is dependent on a complex between the E2s UBC6 and UBC7 which must be membrane bound for MATα2 degradation (Chen *et al.*, 1993; see 1.7.1.6), and a proteasomal subunit (Hochstrasser, 1994). Both *Deg1* and *Deg2* signals appear specific to this protein.

1.5.2.3 The Destruction Box:

Another proteolytic signal for the ubiquitin system is the "destruction box". This is a conserved motif of nine amino acids with the consensus RXALGXIXN (where X is any amino acid). It is required for degradation of mitotic cyclins via the

ubiquitin pathway (Glotzer *et al.*, 1991). All mitotic cyclins characterised have this signal, and Lys-rich sequences C-terminal to it, suggesting these may act as the site of ubiquitination. The proteins of the cyclosome recognize the destruction box.

Other proteins such as CDC25p, the Ras exchange factor, are also destabilised via sequences similar to cyclin destruction boxes (Kaplon and Jacquet, 1995), but these may not be recognised by the cyclosome.

1.5.3 MONOUBIQUITINATION AND UBIQUITIN-LIKE DOMAINS:

The requirement for a degradation signal can be bypassed if one ubiquitin molecule is already attached to the substrate protein. In engineered fusion proteins containing ubiquitin, the ubiquitin acts as a target for further ubiquitination, dependent on the E2 UBC4, and leads to rapid degradation of the multiubiquitinated protein (Johnson *et al.*, 1992). The UFD (ubiquitin fusion degradation) pathway, recognises fused ubiquitin as a degradation signal, and controls the ubiquitin-dependent degradation of these fusion proteins (Johnson *et al.*, 1995). Lys29-linked multiubiquitin chains may be important for this pathway.

Some proteins have ubiquitin-like domains at their C-termini, for example BAG-1 (Takayama *et al.*, 1995). BAG-1 is a short-lived protein, and this domain may act, like fused ubiquitin, as a signal for ubiquitin-dependent degradation.

1.5.4 MULTIPLICITY OF E2 ENZYMES:

There are many E2 enzymes present within a eukaryote (see 1.7), perhaps because each one is responsible for the ubiquitination, and subsequent degradation, of a subset of cellular proteins. Different E2s may be induced during differentiation of cells in order to degrade specific proteins (Wefes *et al.*, 1995), and different classes of artificial degradation signal are recognised by different sets of E2s (Sadis *et al.*, 1995).

The C-termini of E2s are often necessary for recognition of target proteins. When the C-terminal tail of *S.cerevisiae* E2 RAD6 is deleted, yeast are defective in sporulation (Morrison et al., 1988). Gosink and Vierstra (1995) made chimeric E2s by fusing specific protein-binding peptides to their C-termini. They specifically recognised and ubiquitinated their binding partners, and one protein was even degraded via the ubiquitin pathway. This does not actually show C-termini are

responsible for substrate recognition, as the same results may have been obtained by fusing the peptides to the N-termini, and it is not known that C-terminal domains of normal E2s are protein binding domains, but it again suggests C-terminal domains are involved in substrate selection. Also, a chimeric E2 of *S.cerevisiae* RAD6 linked to the C-terminal domain of another E2, CDC34, can perform the functions of CDC34 (Kolman et al., 1992).

1.5.5 DISCUSSION:

All proteins require an available Lys residue for ubiquitin attachment to initiate degradation via the ubiquitin pathway. Signals for degradation are probably all bipartite, requiring the Lys in spatial proximity to the other part of the signal. The N-terminus may be the degradation signal for foreign proteins in the cell, and proteins in the wrong compartment within the cell. Abnormal or damaged proteins may be recognised by exposure of hydrophobic domains that are usually hidden within the protein. Recognition of lysosome by the ubiquitin pathway requires reduction of a disulphide bond which results in localised unfolding at the C-terminus, exposing the putative acceptor Lys (Hill *et al.*, 1993).

Proteins which need to be precisely regulated with respect to half-life, are probably recognised because they contain binding sites for specific E2 and/or E3 enzymes.

1.6 UBIQUITIN ACTIVATING ENZYMES:

Ubiquitin activating enzymes, or E1s, (review: Hershko and Ciechanover, 1992) are abundant proteins of the cytosol and nucleus (Ciechanover *et al.*, 1982; Cook and Chock, 1991a.), and mediate the first step in the ubiquitin pathway. Genes encoding E1s have been cloned from various organisms, and appear to encode proteins with nucleotide-binding domains, matching the consensus GlyXGlyXXGly (McGrath *et al.*, 1991). This is probably the site where ATP is bound in ubiquitin activation reactions. E1 enzymes act as homodimers (Ciechanover *et al.*, 1982), and also form complexes with individual E2 enzymes. The active site Cys of an E1, which becomes covalently linked to ubiquitin, is found in a region with low homology to several E2 enzymes (Hershko and Ciechanover, 1992).

1.6.1 YEAST UBIQUITIN ACTIVATING ENZYMES:

UBA1, a *Saccharomyces cerevisiae* gene, was cloned by McGrath *et al.* (1991), and shown to encode a 114 kDa E1. The protein contains a nuclear targetting signal, and is 53% identical to the E1 protein encoded by the gene at the human A1S9 locus, and 45% identical to wheat UBA1 protein, showing UBA1 homologues are strongly conserved in evolution. The *UBA1* gene of *S.cerevisiae* is essential for viability of both spores and vegetative cells.

As an E1 is necessary for all ubiquitinations, and *UBA1* is an essential gene, it was thought to be the only *S.cerevisiae* E1. However, a second gene encoding a protein similar to UBA1 has been cloned and named *UBA2* (Dohmen *et al.*, 1995). The proteins have extensive similarity both in the putative ATP-binding sites, and in the region of the active site Cys. UBA2 is located mainly in the nucleus and is an essential protein. *UBA1* and *2* cannot complement each other, even when subcellular localisation is altered by mutagenesis, implying that they each perform distinct essential functions (Dohmen *et al.*, 1995). The putative active site Cys of UBA2 is essential for viability, but, as yet, no E1 activity has been shown for the protein.

1.6.2 PLANT UBIQUITIN ACTIVATING ENZYMES:

Ubiquitin activating enzymes are encoded by a multigene family in wheat (Hatfield and Vierstra, 1989; Hatfield *et al.*, 1990), dispelling the belief that only one E1 would be present in each eukaryote. However, wheat is a hexaploid, so if there is one E1 per diploid genome, three would be expected in wheat, and three different genes are found, encoding three E1 proteins: *UBA1* and *2* which are almost identical, and *UBA3* which is significantly different (Hatfield and Vierstra, 1992). All 3 catalyse ATP-dependent activation of ubiquitin, and the active site Cys has been identified in UBA1. Domains of homology between the proteins are present for the sites of ubiquitin linkage, nuclear localisation and nucleotide binding. An E1 has also been found in *Arabidopsis* (Leyser *et al.*, 1993), and is encoded by an auxin-resistance gene.

1.6.3 MAMMALIAN UBIQUITIN ACTIVATING ENZYMES:

There are multiple E1 enzymes in mammals, possibly due to the increase in number of cell types compared to yeast. If some specificity for substrate selection for

ubiquitination resides in E1s, then a different subset of proteins could be degraded in each differentiated tissue by virtue of tissue-specific ubiquitin activating enzymes.

In humans a *UBA1* homologue, from the A1S9 locus, was cloned by Zachsenhaus and Shenin (1990), and another E1 which appeared to have general functions was cloned by Handley *et al.*, (1991). The second E1 has a single mRNA, seen in all tissue and cell lines tested. However, it encodes two proteins with different subcellular locations: one is found mainly in the cytoplasm, and the other exclusively in the nucleus (Handley-Gearhart *et al.*, 1994)

Two human E1 proteins, $E1_{110K}$ and $E1_{117K}$, discovered in HeLa cells, were found to differ in proportion to one another depending on cell type (Cook and Chock, 1992). They correspond in size to the E1s observed by Handley-Gearhart *et al.*, (1994), and are probably the same proteins. There is also a testes specific E1 (Cook and Chock, 1992), probably the homologue of the mouse E1 which is a candidate for the spermatogenesis gene, and is found on the Y chromosome (Kay *et al.*, 1991; Mitchell *et al.*, 1991).

1.6.4 DROSOPHILA UBIQUITIN ACTIVATING ENZYMES:

A *UBA1* homologue in *Drosophila*, *Duba1*, has been cloned in our laboratory by Dr P. zur Lage. Fragments of the gene were amplified by the polymerase chain reaction, using primers annealing to the nucleotide binding motif, and the active site Cys region. The gene, and its encoded protein, have not yet been characterised, but so far the sequence shows 66% identity to the human A1S9 gene.

1.6.5 E1 AND THE CELL CYCLE:

The first indication that ubiquitin activating enzymes were involved in cell cycle progression was the discovery that a mouse cell line, t.s.85, mutant for the cell cycle, was temperature sensitive due to a thermolabile E1 (Finley *et al.*, 1984). When t.s.85 cells are shifted to the non-permissive temperature, cell cycle arrest occurs at early G2 phase, and mitosis is completely inhibited. This could be due to failure to degrade cell cycle controlling proteins. E1s have another putative cell cycle role in histone ubiquitination. Histones are ubiquitinated in interphase (see 1.3.2.), and rapidly deubiquitinated at metaphase when chromosomes condense. E1s are associated with condensed chromosomes during mitosis (Cook and Chock, 1991b.), perhaps so that they are ready to ubiquitinate histones for rapid decondensation after

telophase. Grenfell *et al.* (1994) also saw cell cycle dependent nuclear localisation of ubiquitin activating enzymes. A differential distribution of eight E1 isoforms was found throughout the cell cycle, some of which were assumed to be inactive. Conversion of inactive E1 to active forms may be another mechanism for regulation of ubiquitin-mediated proteolysis.

1.7 UBIQUITIN CONJUGATING ENZYMES:

Ubiquitin conjugating enzymes, or E2s (reviews: Jentsch *et al.*, 1990; Jentsch, 1992), take activated ubiquitin from E1 enzymes and transfer it to the substrate with or without the aid of an E3. Sequence comparisons revealed all E2s have a conserved central domain of 16kDa, the UBC domain (ubiquitin conjugating domain; figure 1.6). This domain exhibits at least 35% identity in all E2s and includes the essential central active site Cys residue, required for thiol-ester linkage to ubiquitin. Sequences bracketing this Cys are highly conserved, as is a proline-rich element located N-terminal to the conserved Cys. At the N-terminal end of the UBC-domain is a conserved domain rich in basic residues, thought, due to deletion analysis, to be the region where E1 binds (Sullivan and Vierstra, 1991). X-ray crystallographic studies on UBC1 of *Arabidopsis thaliana* agree with this being the E1 binding region (Cook *et al.*, 1992).

E2s can be divided into three classes with respect to extensions to the UBC domain (Jentsch *et al.*, 1990). Class I consist almost entirely of the UBC domain, and are barely able to transfer ubiquitin from the E1 to test proteins *in vitro*, suggesting they require E3s for substrate recognition. C-terminal extensions to the UBC domain are present in Class II E2s, and they contribute to the substrate specificity of these enzymes. E2 target recognition can be redefined by engineering E2s to contain specific protein binding peptides fused to their C-termini (Gosink and Vierstra, 1995). This could be a useful tool in biological research, allowing the depletion of a specific cellular protein. C-terminal extensions can also function in cellular localisation. Class III E2s have N-terminal extensions to the UBC domain, but no C-terminal ones (Jentsch *et al.*, 1991). The function of N-terminal extensions is unknown.

Typically, ubiquitin conjugating enzymes are small proteins of 14-32kDa (Hershko and Ciechanover, 1992), and E3s are much larger (100-300kDa). An unusually large E2 of 230kDa is present in rabbit reticulocytes, and appears to

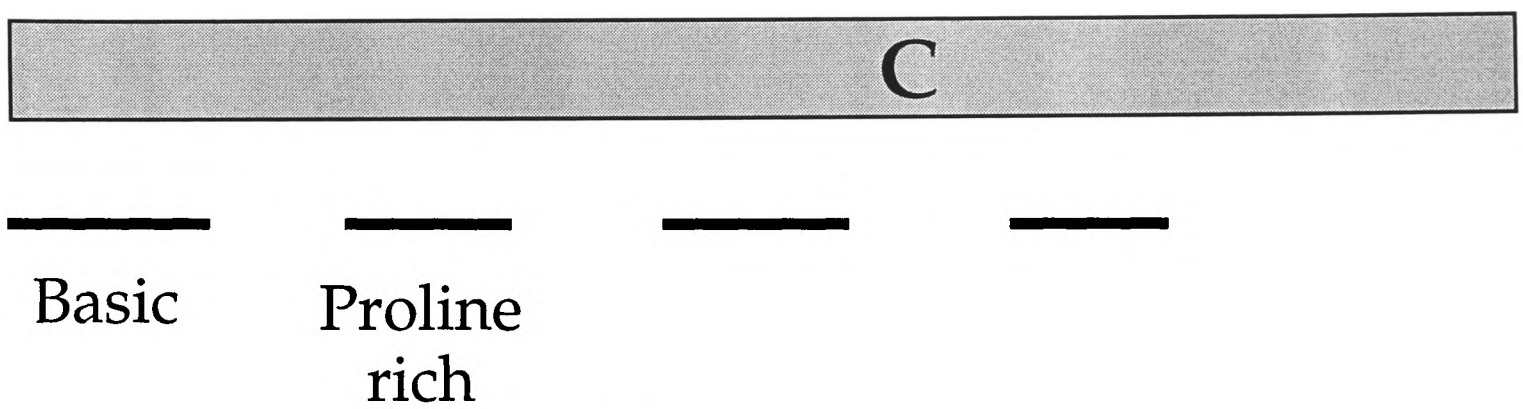


Fig. 1.6 The UBC domain of E2 enzymes.
The central active site Cys is shown, and conserved regions are indicated by the dark lines.

catalyse ubiquitination via an intramolecular thiol relay mechanism, suggesting it functions as an E2-E3 hybrid (Berleth and Pickart, 1996).

E2s have been most thoroughly studied in *S.cerevisiae*.

1.7.1 *SACCHAROMYCES CEREVISIAE* E2S:

According to several sources, twelve E2s have been found so far in yeast, although it appears only ten have been published. Table 1.2 shows the diverse functions of the published yeast E2s.

1.7.1.1 *UBC1, UBC4, UBC5* and homologues:

UBC4 and *UBC5* genes were cloned by Seufert and Jentsch (1990), and the encoded proteins mediate selective protein degradation of short-lived and abnormal proteins. *UBC4* and 5 are closely related in sequence, and complementary in function. When both genes are mutant, there is a slow growth phenotype. Both are induced by high temperature, and double mutants are inviable at high temperature (Seufert and Jentsch, 1990). These enzymes are the major E2s for stress response ubiquitin-dependent degradation.

A third member of this functional class of E2s is *UBC1* (Seufert *et al.*, 1990). All three are functionally overlapping, and together constitute an enzyme family essential for cell growth and viability, due to their functions as the major E2s for bulk degradation of short-lived and abnormal proteins. Unlike *UBC4* and *5*, *UBC1* is not induced by heat shock, but overexpression of *UBC1* can improve growth of *ubc4ubc5* mutants and restore growth of these mutants under stress conditions such as elevated temperatures (Seufert *et al.*, 1990).

Yeast cells mutant for *UBC1* show a moderate slow growth phenotype, but are markedly impaired in growth after germination (Seufert *et al.*, 1990). The degradation of certain proteins may be crucial at this point in yeast life cycle. A similar, but more severe, phenotype is seen for *ubc1ubc4* double mutants, but *ubc1ubc5* mutants show the same phenotype as *ubc1* mutants, proving *UBC5* is dispensible as long as *UBC4* is present.

The UBC4 enzyme is monoubiquitinated *in vivo*, dependent on the expression of active UBC4, indicating that there is a homodimer interaction resulting in ubiquitination (Gwozd *et al.*, 1995). E2-E2 homo- and hetero- interactions have

GENE	E2 CLASS	FUNCTION
UBC1	II	Vital functions with *UBC4* and *UBC5*
UBC2/RAD6	II	DNA repair, induced mutagenesis, sporulation, N-end rule pathway.
UBC3/CDC34	II	G1-S cell cycle control
UBC4	I	Bulk degradation of short-lived and abnormal proteins
UBC5	I	Vital functions with *UBC1* and *UBC4*
UBC6	II	E.R. membrane protein, secretory pathway functions
UBC7	I	Confers cadmium resistance
UBC8	II	No known mutant phenotype
UBC9	I	Probably involved in S and M phase B-type cyclin degradation
UBC10/PAS2	II	Peroxisome biogenesis

Table 1.2 *Saccharomyces cerevisiae* ubiquitin conjugating enzymes.

been reported elsewhere (e.g. Chen *et al.*, 1993), and may serve to increase the repertoire of substrate specificity in the ubiquitin system. Multiubiquitination of a protein by a complex of two E2 enzymes (homo- or hetero- dimers) could be a general mechanism, and two ubiquitin moieties can be added at a time due to the two ubiquitin binding sites present.

Homologues of *UBC1, 4* and *5* have been found in *Arabidopsis* (see 1.7.2), wheat (Girod and Vierstra, 1993), *C.elegans* (Chen *et al.*, 1993), *Drosophila* (see 1.7.3), and humans (Jensen *et al.*, 1995). This implies that this is a highly conserved gene family, whose encoded enzymes perform essential functions in all eukaryotic cells. The proteins are so functionally conserved across evolution, they can complement yeast mutant for these genes. Higher eukaryotes have more members of this E2 subfamily than yeast. In humans, genes *UbcH5A-C* (Jensen *et al.*, 1995) and *UbcH6* (Nuber *et al.*, 1996) are members of the *UBC4/5* family, and Southern transfer experiments show there are likely to more members (Jensen *et al.*, 1995).

1.7.1.2 *UBC2/RAD6* and homologues:

The *RAD6* gene of *S.cerevisiae* is required for postreplication repair of damaged DNA, for induced mutagenesis and for sporulation (Reynolds *et al.*, 1985). It encodes a class II ubiquitin conjugating enzyme (Jentsch *et al.*, 1987) with a highly acidic C-terminal extension containing a tract of 13 consecutive aspartate residues. Ubiquitination of chromosomal proteins by RAD6, which is a nuclear protein (Watkins *et al.*, 1993), may be responsible for specific alterations in chromatin structure required for DNA repair and sporulation.

Mutating the active site Cys88 of RAD6 to Val results in an enzyme which is unable to bind to ubiquitin. The mutant phenotype seen was the same as that of a *rad6* null allele (Sung *et al.*, 1990). This suggests all the biological functions of RAD6 require its E2 activity. The carboxyl-terminal acidic domain extends freely as a tail to the globular core of RAD6 (Morrison *et al.*, 1988) and is essential for multiubiquitination of histones *in vitro* (Sung *et al.*, 1988) and sporulation (Morrison *et al.*, 1988; Sung *et al.*, 1988), but not for DNA repair functions. The polyacidic tail may recognise and bind to basic histones by ionic interactions.

RAD6/UBC2 has been implicated in the N-end rule pathway (see 1.5.1). The ubiquitination of N-end rule substrates requires *UBC2* and the E2 is physically associated with UBR1 (N-recognin) (Dohmen *et al.*, 1991). The yeast *UBC2* can also function as an N-end rule-mediating ubiquitin-conjugating enzyme in a heterologous

cell-free system such as rabbit reticulocyte lysates (Sung *et al.*, 1991). The physical stability of UBR1-UBC2 interaction was seen by Madura *et al.*(1993) to require the C-terminal tail of UBC2, and Watkins *et al.* (1993) showed it required the nine amino terminal amino acids, so both may be involved. An 170 residue C-terminal domain of UBR1 binds UBC2 (Madura *et al.*, 1993).

Among the spontaneous mutations found in *rad6* mutants are insertional mutations due to transposition of Ty retrotransposons (Picologlou *et al.*, 1990). There is a more than 100-fold increase in spontaneous retrotransposition, though there is no increase in Ty message level. This could be due to alterations in chromatin structure of *rad6* cells, allowing greater access for transposition. Ty transposition does not account for the entire increase in spontaneous mutation in *rad6* mutants. Enhancement of G.C to T.A transversion, and both types of base-pair transition account for most of the increase in mutations (Kang *et al.*, 1992), whilst mismatch repair is unaffected. A similar phenotype to that of *rad6* mutants is seen in *rad18* mutants, and the encoded proteins may function by interacting with one another (Kang *et al.*, 1992).

RAD6 homologues have been found in *Drosophila* (see 1.7.3.4), rabbits (Wing *et al.*, 1992), *S.pombe* (Reynolds *et al.*, 1990), and humans (Koken *et al.*, 1991b.; Kaiser *et al.*, 1994). These proteins are very similar to RAD6, though all lack the C-terminal acidic domain. They can all functionally complement *rad6* mutants for DNA repair defects, but not for sporulation which requires the polyacidic tail.

S.pombe has one *RAD6* homologue, *rhp6* (Reynolds *et al.*, 1990), but humans have at least three (Koken *et al.*, 1991b.; Kaiser *et al.*, 1994) showing higher eukaryotes again have multiple E2 enzymes for a particular function. *UbcH1* (Kaiser *et al.*, 1994) has not been fully characterised. *HHR6A* and *6B* are 95% identical (Koken *et al.*, 1991b.) and the locations of their genes within the human genome have been found (Koken *et al.*, 1992). *HHR6A* and *6B* are both expressed in all mammalian tissues and cell types examined (Koken *et al.*, 1996), but have elevated levels in testis. Like RAD6, both proteins are located in the nucleus, and under the electron microsope are seen associated with transcriptionally active regions in euchromatin (Koken *et al.*, 1996). The *HHR6* genes may have overlapping functions related to DNA repair.

The RAD6 protein is a highly conserved protein, probably present, and performing a similar role, in all eukaryotic cells.

1.7.1.3. *UBC3/CDC34* and homologues:

CDC34 is an essential gene in *S.cerevisiae*, necessary for the transition from G1 to S phase. Temperature sensitive (t.s.) *cdc34* mutants arrest with numerous elongated buds at the nonpermissive temperature. The spindle pole body duplicates, but fails to undergo the separation required for spindle formation. *CDC34* was found to encode a class II ubiquitin conjugating enzyme and was renamed *UBC3* (Goebl *et al.*, 1988). It has substantial similarity to *RAD6* both within the UBC domain, and the polyacidic C-terminal extension. The C-terminal domain is 125 amino acids long, and the 39 residues adjacent to the catalytic domain are necessary and sufficient for full cell cycle functions (Ptak *et al.*, 1994).

When the active site Cys of CDC34 is mutated to Ser, cell growth is blocked, but no multi-budded phenotype is seen (Banerjee *et al.*, 1995). The block is relieved by overexpression of wild type CDC34, so the mutant probably sequesters CDC34 substrates. This shows *CDC34* cell cycle functions are dependent on it functioning as an E2 enzyme.

The *cdc34* mutant phenotype can be partially suppressed by overexpression of wild type ubiquitin (Prendergast *et al.*, 1995). This may be due to CDC34 catalysing its own ubiquitination, as a homodimer, via an intramolecular transfer of its thiol-ester linked ubiquitin to a Lys (Banerjee *et al.*, 1993). This is similar to the homodimer interaction of UBC4 except CDC34 becomes polyubiquitinated, suggesting one function of the enzyme is to target its own degradation. The physical interaction between CDC34 monomers is dependent on the same region of the C-terminal extension necessary for cell cycle functions (Ptak *et al.*, 1994). When CDC34 catalytic and tail domains are expressed separately, they only partially function (Silver *et al.*, 1992). The cell cycle functions of CDC34 are dependent on the ability of CDC34 monomers to interact with each other.

When *S.cerevisiae* cells reach a critical size, they initiate bud formation, spindle pole body duplication, and DNA replication almost simultaneously. This is the region of the cell cycle where *CDC34* is necessary. All three events require activation of the CDC28 protein kinase by G1 cyclins CLN1, 2, and 3. CLN3 is a very unstable protein, due to a C-terminal PEST sequence (Tyers *et al.*, 1992). It associates with CDC28 to form an active kinase complex that phophorylates CLN3 itself. A mutant CDC34 enzyme is seen to dramatically increase CLN3 associated kinase activity, but does not affect CLN3 half-life. However, CLN2, which also

binds to CDC28 kinase, stimulates kinase activity and becomes phophorylated, is ubiquitinated and degraded in a *CDC34* dependent manner (Deshaies *et al.*, 1995).

DNA replication also requires activation of CDC28 kinase by B-type cyclins CLB1-6 (Schwob *et al.*, 1994). Mutants for all six B-type cyclins arrest as multi-budded G1 cells resembling *cdc34* t.s. mutants. Schwob *et al.* (1994) found *cdc34* mutants could not enter S phase because they fail to mediate the degradation of $p40^{SIC1}$, a potent inhibitor of CLB forms of the CDC28 kinase. G1 to S phase transition therefore requires *CDC34* mediated degradation of $p40^{SIC1}$, a protein which appears at the end of mitosis, disappears before S-phase, and is a cyclin-specific inhibitor of CDC28 kinase.

A key component of the yeast centromere kinetochore is the protein complex CBF3. *CBF2* encodes a subunit of CBF3, and CBF2 protein is another substrate for *CDC34* mediated ubiquitination (Yoon and Carbon, 1995), implying ubiquitination of a kinetochore protein plays a regulatory role in kinetochore function.

Kornitzer *et al.* (1994) demonstrated *CDC34* is also necessary for the rapid turnover of GCN4, a regulator of amino acid biosynthesis. This is a non-cell cycle function for *CDC34* indicating it is a multifunctional regulator.

The first ubiquitin conjugating enzyme suppressor, *UBS1*, has been cloned and is a general positive regulator of CDC34 (Prendergast *et al.*, 1996). When overexpressed, UBS1 suppresses mutant *cdc34* cell cycle defects, as well as the inability to degrade GCN4. Deletion of *UBS1* in a *cdc34* mutant accentuates the cell cycle defect. UBS1 may regulate CDC34 activity by independently interacting with a repressor of CDC34 via regions of similarity found between CDC34 and UBS1 (Prendergast *et al.*, 1996). CDC34 is phosphorylated and ubiquitinated (Goebl *et al.*, 1994), so activation/repression of the E2 could act by posttranslational modification.

CDC34 homologues have been found in rabbit (Haas *et al.*, 1993) and humans (Plon *et al.*, 1993). The human homologue is necessary for late G1 to S transition, and can functionally substitute for *CDC34* (Plon *et al.*, 1993). Southern transfer experiments showed the human*CDC34* DNA cross hybridised to DNA from other eukaryotes, suggesting this E2 has been highly conserved across evolution.

1.7.1.4 *UBC2* and *UBC3* chimeric E2s:

When a chimeric E2 consisting of the RAD6 catalytic domain, and the 125 amino acids comprising the C-terminal tail of CDC34 was expressed in yeast, it

performed *CDC34* functions and complemented the growth defects, U.V. sensitivity and sporulation defects of *rad6* mutants (Kolman *et al.*, 1992). The 74 amino acids of the CDC34 tail neighbouring the catalytic domain were also able to perform these functions in the chimeric protein (Kolman *et al.*, 1992; Silver *et al.*, 1992). The cell cycle functions of *CDC34* are probably partly encoded by the tail domain, and partly in a conserved region of the catalytic domain shared between RAD6 and CDC34. The CDC34 catalytic domain cannot substitute for the DNA repair functions of *RAD6* (Silver *et al.*, 1992). This again shows substrate specificity of E2 enzymes can reside in C-terminal extensions to the UBC domain.

1.7.1.5 *UBC6*:

UBC6 is a class II E2 enzyme with a membrane anchor signal in its C-terminal extension. The encoded protein localises to the endoplasmic reticulum (E.R.) with the catalytic domain facing the cytosol (Sommer and Jentsch, 1993). Null mutants of *UBC6* are viable, but suppress protein translocation defects of *sec61* mutants. *SEC61* encodes a necessary component of a multisubunit protein translocation apparatus of the E.R.. The *sec61* mutant phenotype is only seen when UBC6 is expressed (Sommer and Jentsch, 1993). *UBC6* may mediate ubiquitin-dependent proteolysis of E.R. membrane proteins, and *sec61* translocation defects may be due to proteolysis of the other components of the mutant translocation apparatus.

Degradation of E.R. membrane proteins may be related to E.R. protein degradation systems and may be a general function of the ubiquitin system. *UBC6* could be responsible for mediating degradation of various aberrant membrane proteins that are degraded in the E.R..

1.7.1.6 *UBC7*:

UBC7 shows similarity to the *UBC1*, *4*, and *5* group of E2s, but *ubc7* mutants are viable and grow at wild type rates (Jungmann *et al.*, 1993). *UBC7* mediated functions are not required under normal growth conditions, but *ubc7* mutants are specifically cadmium hypersensitive (Jungmann *et al.*, 1993). Wild type cells require a functional proteasome for cadmium resistance, and *UBI4*, *UBC5* and *UBC7* are all cadmium inducible genes. This suggests that a major reason for cadmium toxicity is the formation of abnormal proteins, and that the ubiquitin pathway is induced to

degrade these proteins. In order to mediate cadmium resistance, it may be necessary for*UBC7* to be active to degrade abnormal proteins.

UBC6 and UBC7 associate as a complex to target *Deg1* dependent degradation of MATα2 (Chen *et al.*, 1993). The interaction between the E2 enzymes does not require the UBC6 membrane anchor, but membrane association of the complex is required for *Deg1*-mediated proteolysis. This shows that there can be an overlap in substrate specificity among diverse E2s, and one mechanism of substrate selection may be by combinatorial association of different ubiquitin conjugating enzymes. UBC7 proteins may also interact with one another (Chen *et al.*, 1993).

1.7.1.7 *UBC8*:

UBC8 (Qin *et al.*, 1991) has no detectable mutant phenotype. It has an acidic C-terminal extension to the UBC domain, as does its human homologue, *UbcH2* (Kaiser *et al.*, 1994). The UBCH2 C-terminal domain is thought to interact with substrates for ubiquitination (Kaiser *et al.*, 1995). *UBC8* may, like *UBC7*, be necessary for conferring resistance to specific toxic substances which produce abnormal proteins.

1.7.1.8 *UBC9* and cell cycle control:

B-type cyclin degradation in yeast requires the activity of the essential, nuclear ubiquitin conjugating enzyme UBC9 (Seufert *et al.*, 1995). Repression of UBC9 synthesis prevents cell cycle progression at G2 or early M phase, resulting in large budded cells with one nucleus, a short spindle and replicated DNA. Both CLB5, an S-phase cyclin, and CLB2, an M-phase cyclin, are stabilised in *ubc9* mutants (Seufert *et al.*, 1995). CLB5 is short-lived throughout the cell cycle in wild-type cells, and CLB2 is stable during S and M phase, but is extremely short-lived in pre-start G1 cells. UBC9 protein is necessary at different points in the cell cycle to degrade different B-type cyclins. Therefore, cell cycle specific cyclin proteolysis must require distinct degradation signals, probably the "destruction boxes", and regulated interaction with ubiquitin system enzymes.

The *hus5* gene of *S.pombe* is probably a homologue of UBC9 (Al-Khodairy *et al.*, 1995). Deletion mutants of *hus5* have very slow growth and exhibit high levels of abortive mitoses. Wild type *hus5* may be involved in chromosome segregation.

Entry into S phase, separation of sister chromatids during anaphase, and exit from mitosis all require the degradation of specific proteins via ubiquitin-dependent proteolysis. Cell cycle control by ubiquitin is reviewed in Pines (1994), Deshaies (1995b.) and Murray (1995).

1.7.1.9 *UBC10/PAS2*:

Another yeast ubiquitin conjugating enzyme is encoded by the gene *PAS2*, renamed *UBC10*, and is required for peroxisome biogenesis (Wiebel and Kunau, 1992). Peroxisomes are small membrane-bounded organelles that use oxygen to oxidize organic molecules. They contain enzymes to produce and degrade hydrogen peroxide. When a *PAS* gene is mutant, it leads to the absence of functional peroxisomes. Site-directed mutagenesis of the active site Cys of PAS2 resulted in a protein unable to complement *pas2* mutant strains, but still associating with peroxisomes (Wiebel and Kunau, 1992). The C-terminal extension to the UBC domain probably targets PAS2 protein to the peroxisome membrane. There is a crucial role for the ubiquitin-conjugation pathway in peroxisome formation as *pas2* mutants lead to the mislocalisation of peroxisome matrix enzymes.

1.7.2 *ARABIDOPSIS THALIANA* E2S:

More E2s have been cloned in *Arabidopsis thaliana* than *S.cerevisiae*, but their functions have not been well characterised. Genes for sixteen E2s have been cloned, and they can be divided into 6 groups as shown in table 1.3 (Vierstra, 1994). *AtUBC1-7* encode four structurally and functionally distinct subfamilies of E2s (Sullivan and Vierstra, 1991; Girod *et al.*, 1993) and *AtUBC8-12* are members of a multigene family encoding homologues of *S.cerevisiae UBC1*, *4*, and *5* E2s (Girod *et al.*, 1993).

The expression of *AtUBC3*, *8*, and *9* was investigated by Genschik *et al.* (1994). All three are under control of complex mechanisms and are differentially regulated with respect to the lifetime of the cell, different organs in the plant, and development of the plant. None of them have coordinated expression with ubiquitin.

1.7.3 *DROSOPHILA* E2S:

The *Drosophila* E2s found so far are shown in figure 1.7.

GENE	YEAST HOMOLOGUE
UBC 1, 2	*UBC2 / RAD6*
UBC 3	*CDC34*
UBC 4, 5, 6	*UBC8*
UBC 7, 13, 14	*UBC7*
UBC 8, 9, 10, 11, 12	*UBC4, 5*
UBC 15, 16	?

Table 1.3 *Arabidopsis thaliana* E2 enzymes (Vierstra, 1994).

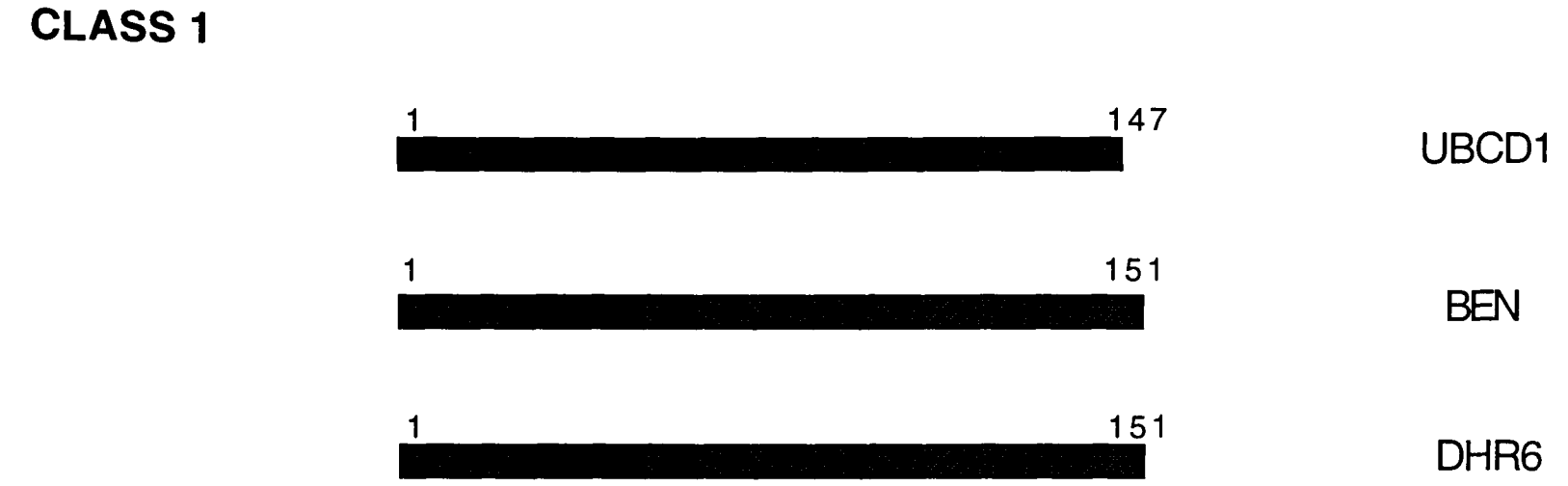


Fig. 1.7 *Drosophila melanogaster* E2 enzymes arranged to show the different structural classes. UBC domains are shaded.

1.7.3.1 *UbcD1*:

UbcD1 was cloned by PCR amplification with degenerate primers (Treier *et al.*, 1992). The encoded protein is a class I E2, and is very similar in sequence to *UBC4* and *UBC5*. When *UBC4* is precisely replaced with the *UbcD1* ORF in a *ubc4ubc5* double mutant strain, it rescues the growth defects and temperature sensitivity (Treier *et al.*, 1992). *UbcD1* mediates the multiubiquitination of proteolytic substrates, as most of the high molecular weight ubiquitin conjugates, absent in *ubc4ubc5* double mutants, are restored in the UBCD1 expressing cells. UBCD1 can also degrade abnormal proteins produced by the presence of canavine.

UBCD1 is an E2 probably responsible for bulk-degradation of short-lived and abnormal proteins in *Drosophila*. It has a transcript of 1.5kb (Treier *et al.*, 1992, results not shown), and maps to 88D in the *Drosophila* genome (Matuschewski *et al.*, 1996).

1.7.3.2 *UbcD2* and Other Class III E2s:

The *UbcD2* gene was also cloned by PCR amplification with degenerate primers, and encodes a class III E2 with an N-terminal extension to the UBC domain (figure 1.7). Class III E2s form a novel family of ubiquitin conjugating enzymes, all from higher eukaryotes, which are related in function and sequence to yeast *UBC4/5* (Matuschewski *et al.*, 1996). The UBC domains of these enzymes (encoded by *UbcD2* in *Drosophila, UbcM2* and *M3* in mouse, and *UbcH6, 8* and *9* in humans) are 94% identical. The amino-terminal extensions show little sequence similarity, except *UbcH9* and *UbcM2* which are homologues. This E2 family has at least four distinct members.

UBC domains of class III E2s are approximately 64% identical to that of UBC4, as opposed to *UbcD1*, the *Drosophila UBC4* homologue,which is 80% identical. Class II E2s, with or without the N-terminal domain, can partially complement *ubc4ubc5* mutants (Matuschewski *et al.*, 1996), so may have some substrates in common.

No homologues to these genes have been found in yeast, and, as their UBC domains are so conserved within the group, it is likely this family evolved late and is unique to multicellular organisms. The significance of N-terminal extensions is unknown.

According to Matuschewski *et al*. (1996) *UbcD2* has a 1.5kb transcript, and maps to 32A/B in the *Drosophila* genome.

1.7.3.3 *bendless/UbcD3*:

The *bendless* (*ben*) gene was first discovered as an X chromosome mutation in *Drosophila melanogaster,* which alters patterns of connections in the central nervous system (C.N.S.; Thomas and Wyman, 1984).

The giant fibre (GF) neuron mediates the escape jump response in *Drosophila*. It receives input from the visual and olfactory senses, and upon stimulation drives the tergotrochanter (TTM), dorsal longitudinal (DLM) and dorsoventral muscles (DVM). Mutant *ben* genes affect the neuronal connection between the GF and the TTM motor neuron (TTMmn; Thomas and Wyman, 1984). In ben^- flies the GF fails to bend to make the connection with the TTMmn, and flies are defective in the escape jump response. The DLM is unaffected. The *bendless* gene product appears essential for the formation of a single synapse, and the *ben* mutation causes a defect in the recognition event leading to GF and TTMmn synapse formation (Muralidhar and Thomas, 1993). Mosaic analysis showed that the ben^- phenotype corresponds to a presynaptic GF defect, probably involving a growth cone dysfunction (Euk Oh *et al*., 1994).

Other phenotypes are seen in ben^- mutants:- abnormal grooming behaviour, lack of coordination, failure to climb the sides of culture vials, and failure to fly when dropped from a height (Euk Oh *et al.*, 1994). Also 60% of mutant pupae cannot successfully eclose, and often die during emergence. Some of these phenotypes may be due to visual defects found in ben^- flies (Muralidhar and Thomas, 1993; Euk Oh *et al*., 1994). BENDLESS protein affects photoreceptor axons, which, like GF in ben^- flies, initially project normally, but fail later in their pathways.

The *bendless* gene was cloned (Muralidhar and Thomas, 1993; Euk Oh *et al.*, 1994) and found to encode a class I E2. The same gene was found in a PCR amplification screen for *Drosophila* E2s (Matuschewski *et al.*, 1996) and named *UbcD3.* It locates to 12D1-2 in the *Drosophila* genome, and has two transcripts of 1.2 and 2.0kb (Muralidhar and Thomas, 1993). The BENDLESS protein may represent a distinct subclass of E2 as it is not a homologue of any E2 cloned so far. It appears to be most similar to the *UBC1*, *4*, *5* class of E2s, but is unable to rescue *ubc4ubc5* mutants (Matuschewski *et al*., 1996, results not shown).

One *ben*$^{-}$ mutation was found to be a point mutation, causing a Pro to be changed to a Ser at position 97 (Muralidhar and Thomas, 1993). This Pro residue is found in the same position in all known E2s, and is within the strictly conserved consensus of the UBC active site region. This suggests the *bendless* gene product carries out its functions by acting as an E2 enzyme and mediating ubiquitination of other proteins.

Both *bendless* transcripts are expressed during development, with peak levels during pupation and in adults (Muralidhar and Thomas, 1993, results not shown). RNA *in situ* hybridisations show a low level of uniform expression throughout development, until the eleventh hour of development when a dramatic increase in *ben* expression is observed restricted to the nervous system. Most neurons of the C.N.S. and P.N.S. (peripheral nervous system) express high levels of *ben*. In third instar larvae high levels of expression are detected in developing brain and the ventral nerve cord (Muralidhar and Thomas, 1993). The GF circuit becomes active, and synapses between photoreceptor neurons and their targets form during the last third of pupal development. At this stage, high level *ben* expression is seen in the retina, neurons of the esophogeal ganglia of the brain, where the GF cell body is located, and in the thoracic ganglion (Muralidhar and Thomas, 1993). The fact *bendless* expression is restricted to the nervous system raises the possibility other E2s are differentially expressed and play specific roles in tissue development or function.

As *bendless* functions as an E2 enzyme, normal nervous system development must require specific proteins to be functionally modified or degraded via ubiquitination. In the GF circuit, synaptic connectivity is disrupted. Ubiquitin mediated signals could be necessary for activating or stabilising adhesive interactions allowing the growth cone to extend towards its target.

It has recently been reported that one target of the oncogene MYC in mice is a ubiquitin conjugating enzyme with homology to *bendless* (Bernards, 1996). This suggests similar interactions may occur between BENDLESS and *Drosophila* MYC proteins.

1.7.3.4 *Dhr6*:

Dhr6 is the *Drosophila* RAD6 homologue. It was discovered by homologous probing using the *RAD6* gene (Koken *et al.*, 1991a.), and encodes a class I E2, lacking the polyacidic C-terminal tail of RAD6. *Dhr6* has two transcripts of 1.3 and

2.1kb, differing at their 3' ends due to alternative polyadenylation sites. The *Dhr6* gene is located at 82D in the *Drosophila* genome.

When expressed under the *RAD6* promoter, DHR6 increases the U.V. resistance of a *rad6* deletion strain. It also complements the γ-ray sensitivity and restores the wild type level of U.V. induced mutagenesis (Koken *et al.*, 1991a.). *Dhr6* could not rescue the sporulation defects as it lacks the necessary acidic C-terminal domain. The structure and function of the protein encoded by *Dhr6* has been highly conserved among eukaryotes.

A gene encoding a fifth *Drosophila* E2 has recently been cloned and is a member of the alpha-esterase cluster of genes (Robin *et al.*, 1996).

1.8 UBIQUITIN-PROTEIN LIGASES:

Where a ubiquitin protein ligase (E3) is necessary for ubiquitination of the substrate protein, a thiol-ester linkage of ubiquitin to the enzyme is often, if not always, an intermediate in the reaction. Specificity in ubiquitin conjugation is partly due to the existance of multiple E2s and E3s. E2s are small proteins, and E3s are relatively large (100-300kDa) and specifically and tightly bind protein substrates (Reiss and Hershko, 1990; Scheffner *et al.*, 1993). It seems likely E3s bind to the substrates, and E2s bind to E3s to allow the transfer of ubiquitin. In the large E2$_{230K}$, both E2 and E3 activities reside within the same protein (Berleth and Pickart, 1996), and a thiol relay is seen between the two Cys residues in the protein.

1.8.1 N-END RULE E3S: E3α, E3β AND UBR1:

The first E3s characterised were those involved in recognising the N-terminus of protein substrates for degradation via the "N-end rule" ubiquitin-dependent pathway (see 1.5.1; review: Hershko, 1991).

E3α was isolated from rabbit reticulocytes (Hershko *et al.*, 1983), and purified by affinity chromatography on immobilized Type I or Type II protein substrates for the N-end rule (Reiss *et al.*, 1988). E3α is therefore thought to recognise the amino-terminal residues of proteins. It promotes the addition of multiple ubiquitins to the substrate protein (Hershko and Heller, 1985), and contains specific binding sites for proteins prior to ubiquitination. N-terminal recognition is

based on the specificities of E3α binding sites (Reiss *et al.*, 1988). It has one binding site for Type I basic N-terminal residues, and one for Type II bulky hydrophobic residues. E3β was isolated in the same screen as E3α. It recognizes N-end rule substrates with free, small uncharged N-termini (Heller and Hersko, 1989).

The yeast homologue of E3α was cloned by Bartel *et al.* (1990). The gene, *UBR1*, encodes a protein required for *in vivo* degradation of test proteins with basic or bulky-hydrophobic N-termini, and contains separate binding sites for each. Null *ubr1* mutants are viable, but grow slightly more slowly than wild type cells, and are slightly defective in sporulation. UBR1 has binding sites for and interacts with the UBC2/RAD6 protein (see 1.7.1.2; Madura *et al.*, 1993).

1.8.2 E3S WITH KNOWN SPECIFIC SUBSTRATES:

E3s other than those for N-end rule functions have been discovered. A cyclin specific E3, E3-C, is present in clam embryos as the cyclosome complex or anaphase promoting complex (APC), activated at the end of mitosis by cdc2 kinase (Lahavbaratz *et al.*, 1995). A similar E3 targetting cyclinB destruction in mitosis (M) is present in *Xenopus* embryos (King *et al.*, 1995).

An E3, different both structurally and functionally from all other known ligases, was found in rabbit muscle (Gonen *et al.*, 1996). It was named E3L, and recognises muscle proteins actin, troponin T and MyoD. It may be specific for muscle protein degradation, as it is found in other mammals, but not in wheat. Higher eukaryotes may require many E3 enzymes for differentiation of different tissues.

Calmodulin is monoubiquitinated with the aid of UBC4 and a putative specific E3 (Parag *et al.*, 1993) in *S.cerevisiae*. Ubiquitinated calmodulin does not appear to be degraded, so the putative E3 may be necessary for ubiquitination to modify the activities of the protein.

1.8.3 "HECT" DOMAIN E3S:

The human *E6-AP* (E6 associated protein) gene was discovered as part of the mechanism by which the E6 protein of human papillomaviruses inactivates the p53 tumour suppressor gene. E6-AP is an essential factor in mediating complex formation of E6 and p53 (Huibregtse *et al.*, 1991) and is an E3 enzyme (Scheffner *et*

al., 1993). E6-AP forms a thiol-ester with ubiquitin, dependent on a Cys residue near the C-terminus (Scheffner *et al.*, 1995), which is also necessary for p53 ubiquitination. Ubiquitin is transferred in a "thiol-ester cascade" from E1 to a specific E2, to E6-AP which transfers it to the protein substrate.

Several eukaryotic proteins show similarity to E6-AP at their carboxyl-terminal ends (Scheffner *et al.*, 1995; Huibregtse *et al.*, 1995). This region includes the active site Cys necessary for E6-AP function, and this residue is conserved in all of these proteins. These proteins are known as hect domain proteins (homologous to the E6-AP carboxyl terminus; Huibregtse *et al.*, 1995), and two, a rat 100kDa protein and the yeast RSP5, have been shown to share the ability to form thiol-ester links to ubiquitin (Huibregtse *et al.*, 1995).

Regions of E6-AP-related proteins amino-terminal to the hect domain are generally divergent. In E6-AP this region is involved in binding substrates, suggesting that the hect domain targets specific substrates for ubiquitination due to protein-protein interactions directed by the amino-terminal region.

One member of this family, rat p100, has a region of similarity to a conserved element of poly(A) RNA binding proteins (PABPS) N-terminal to the hect domain (Huibregtse *et al.*, 1995). It has large regions of similarity to another hect protein, the *Drosophila hyperplastic discs* (*hyd*) gene product (see 1.8.4). The regions of similarity include the PABPS element, implying E3 activities of p100 and HYD may target similar proteins for ubiquitination.

The structural and biochemical similarities between E6-AP and the hect domain proteins suggests they represent a class of E3 ubiquitin protein ligases.

1.8.4 *DROSOPHILA* E3S: *HYPERPLASTIC DISCS*:

The *hyd* gene is located at 85E1-10 in the *Drosophila* genome (Mansfield *et al.*, 1994) and was identified as a temperature sensitive (t.s.) mutation causing imaginal disc overgrowth in larvae at the restrictive temperature. Accumulation of the 9.5kb *hyd* transcript during development reflects the expression patterns of HYD protein. They are present at all stages, with high levels at embryonic and pupal stages (Mansfield *et al.*, 1994).

In situ hybridization to endogenous *hyd* transcript shows it is relatively abundant and evenly distributed throughout embryo development (Mansfield *et al.*,

1994). In second and third instar larvae, the transcript becomes restricted to imaginal tissues, and is only present in cells growing by proliferation.

The *hyd* gene appears to be required in the initiation and maintenance of imaginal disc proliferation, as well as the termination of proliferation. Null alleles cause imaginal discs to be smaller than normal, whereas t.s. or hypomorphic alleles cause imaginal disc overgrowth (Amanai and Shearn, 1996).

A hect domain protein is encoded by the *hyd* gene (see 1.8.3). HYD can bind to *bendless* protein, so appears to act as an E3 in *Drosophila* (Amanai and Shearn, 1996). The yeast two-hybrid system was used to find HYD associated proteins, and the D14-3-3 product of the *Leonald* gene was found by this screen (Amanai and Shearn, 1996). The 14-3-3 proteins have regulatory roles in intracellular signalling, and bind to oncogene and proto-oncogene products. 14-3-3 is part of the complex containing Raf which becomes translocated to the plasma membrane in the presence of Ras-GTP as part of the MAPK cascade. Raf is activated by 14-3-3. HYD may control imaginal disc cell proliferation by degradation of the 14-3-3 component in this multiprotein complex. However, binding of D14-3-3 to HYD was only discovered via the yeast two-hybrid system (Fields and Song, 1989), which often produces false positives. Binding has yet to be confirmed with purified proteins and *in vivo*.

1.9 THE 26S PROTEASOME:

The proteasome is the part of the ubiquitin system mediating the degradation of ubiquitin-protein conjugates *in vivo* (reviews: Goldberg, 1995; Jentsch and Schlenker, 1995; Rubin and Finley, 1995). Proteins tagged with multiubiquitin chains are degraded by the proteasome yielding small peptides in an ATP-dependent manner. Proteasomes are abundant (up to 1% of cellular protein) multisubunit particles (review: Peters, 1994) found in the cytosol and nucleus of eukaryotes.

The catalytic core of the 26S proteasome is the 20S protease (see figure 1.8; Peters, 1994), the structure of which has been determined by X-ray crystallography (Löwe *et al.*, 1995). In addition to the 20S protease, protein degradation requires 19S regulatory particles (figure 1.8; De Martino *et al.*, 1994), and together these constitute 26S proteasomes. One subunit of the 19S particle is S5a, mediating recognition of multi-ubiquitin chains (see 1.3.1).

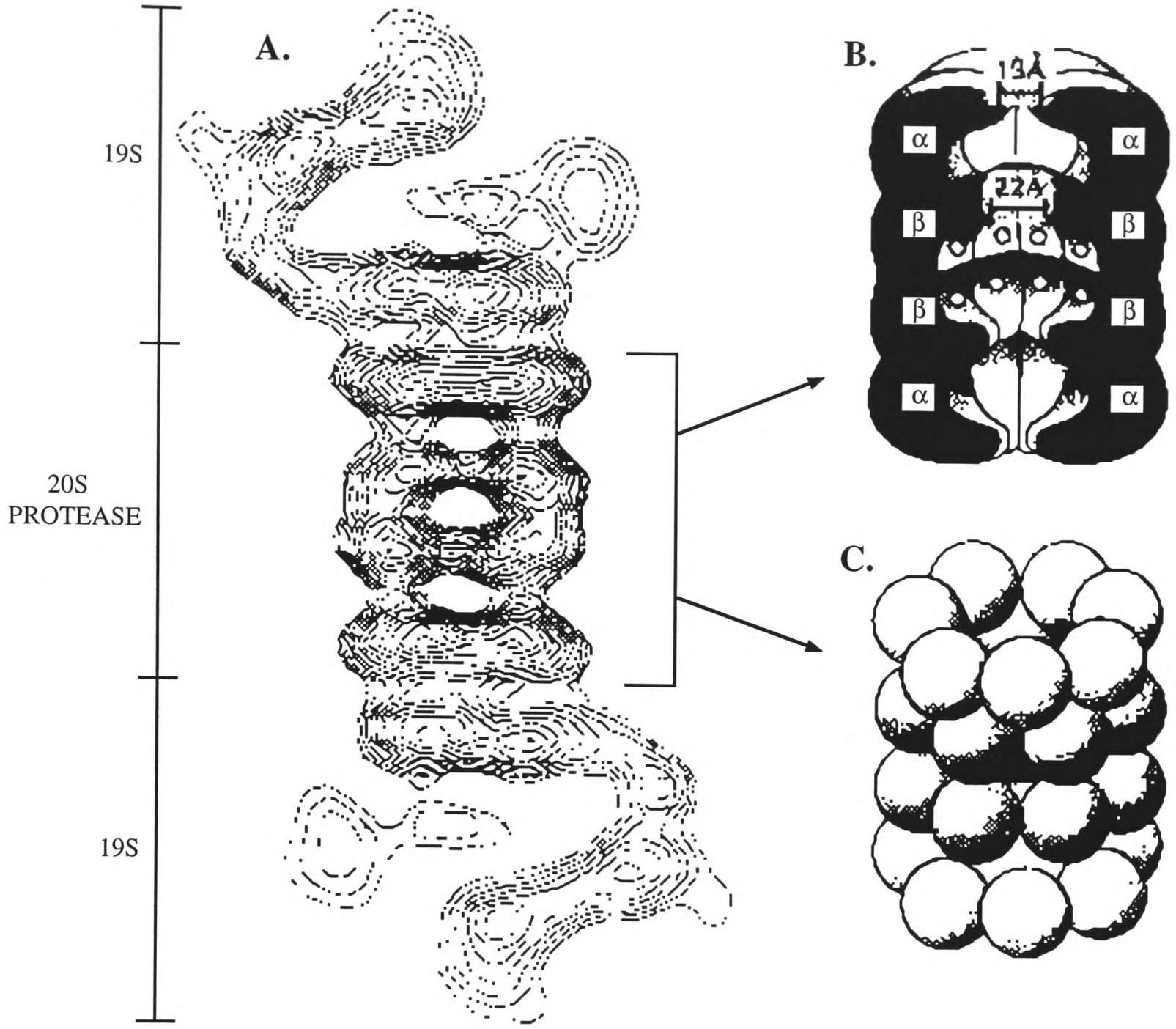


Fig. 1.8 Structure of the 26S proteasome (after Rubin and Finley, 1995).
A. Contour map.
B. Schematic cross section. Circles show the position of peptidase active sites in the *Thermoplasma acidophilum* proteasome.
C.Schematic diagram to show subunit arrangement.

The 20S protease consists of four rings, each of seven subunits, arranged as a hollow cylinder (figure 1.8). Löwe *et al.* (1995) show in the archaebacterium *Thermoplasma acidophilum* proteasome, the subunits are either α or β, and are arranged in outer and inner rings respectively (figure 1.8). In eukaryotes each α and β subunit is encoded by a different gene, and the genes can be differentiated into families. In *S.cerevisiae* there are seven α and seven β genes (Hilt and Wolf, 1995).

Inhibitors of the archaebacterial proteasome bind to β subunits, showing these are proteolytically active (Fenteany *et al.*, 1995). The active site residue is threonine; when it is mutated to Ser in the archaebacterial β subunit, the protease is sensitive to Ser protease inhibitors (Seemüller *et al.*, 1995). In the eukaryotic proteasome there are at least three distinct peptidase specificities (Hilt *et al.*, 1995), and only three of the seven β subunits appear proteolytically active. The eukaryotic proteasome apppears to have separate active sites which cleave after basic, hydrophobic or acidic residues, and there is cooperative interaction between β subunits. In *S.cerevisiae PRE1*, *2*, *3*, and *4* genes encode β subunits (Hilt *et al.*, 1995). *PRE 3* and *4* are necessary for cleavage after acidic residues, and *PRE1* and *2* are responsible for chymotrypsin-like activity (Heinemeyer *et al.*, 1993).

In mammals the three active β subunits are found in distinct basal and inducible forms, and are regulated by cytokines (Fehling *et al.*, 1994). Differences in proteolytic activity and β subunit patterns can be seen in different tissues (Cardozo *et al.*, 1995).

The immune system of higher vertebrates utilizes MHC class I and classII molecules to display peptides, derived from intra- and extra-cellular antigens respectively, at the cell surface for recognition by T cells. The ubiquitin pathway provides peptides for binding to MHC class I molecules (Michalek *et al.*, 1993; Rock *et al.*, 1994), and the genetics of proteasomes and antigen processing is reviewed by Monaco and Nandi (1995). The mediator of the immune response, γ-interferon (IFNγ), enhances antigen presentation, and induces expression of three β subunits including MHC-encoded proteins LMP2 and LMP7 (Driscoll and Goldberg, 1990). These are incorporated into proteasomes in place of homologous normal subunits, resulting in a proteasome which cleaves preferentially after basic and hydrophobic residues. This favours the production of oligopeptides with hydrophobic or basic C-termini, which are preferentially transported into the E.R. by the TAP transporter molecule, also induced by IFN-γ, and bind tightly to MHC class I molecules (Neefjes *et al.*, 1993). Peptide specificity of proteasomes, TAP, and class I molecules is coordinated, and genes for LMP2, LMP7, TAP and class I heavy

chains are linked in the major histocompatibility complex (MHC) (Monaco and McDevitt, 1986).

The active sites of the *T.acidophilum* proteasome face the central cavity (figure 1.8; Löwe *et al.*, 1995). The hydrophobic channel in the middle is approximately 13 Å in diameter, so folded proteins cannot pass through it (Wenzel and Baumeister, 1995). The 19S particle has a channel continuous with that of the proteasome, and contains six distinct ATPase subunits (Dubiel *et al.*, 1995). The interaction of 19S with the proteasome is ATP-dependent (Eytan *et al.*, 1989), and the ATPases may mediate protein unfolding. The muliple ATPases probably have different binding specificities, endowing the 26S proteasome with the capacity to interact with and degrade a diverse range of ubiquitinated proteins.

Ubiquitin chains of conjugates are anchored to 19S subunit 5a (Deveraux *et al.*, 1994), and substrates probably remain anchored until ATPases have unfolded parts of the protein and initiated translocation of the protein into the 20S protease channel. Thus, only ubiquitinated proteins are degraded. The ubiquitin protein is very stable, so must resist the unfolding enzymes in the 19S particle. At a certain stage the multiubiquitin chain must be removed to allow complete translocation of the substrate into the proteasome. Yeast DOA4, a ubiquitin carboxyl-terminal hydrolase (see 1.10), is a likely candidate for this activity, as mutants accumulate multiubiquitinated substrates that appear partially degraded (Papa and Hochstrasser, 1993). The multiubiquitin chains are cleaved to single moieties by isopeptidase T (Hadari *et al.*, 1992; see 1.10), and the unfolded substrate is degraded in the central chamber of the proteasome.

Proteasomes may also mediate the processing of precursors into active proteins. The p50 subunit of NF-κB is generated by a ubiquitin and proteasome dependent reaction, involving the degradation of the C-terminal portion of its precursor (Palombella *et al.*, 1994; see 1.11.1).

The proteasome undergoes changes in intracellular localization during the cell cycle (Kawahara and Yokosawa, 1992; Amsterdam *et al.*, 1993). The observed spatial and temporal distribution pattern during mitosis is like that of cyclins (Pines and Hunter, 1991), suggesting proteasomes play a role in degrading cyclins.

The control of proteasomes may be by exchange of β subunits, which could affect cleavage site specificity. Raising the activity of β subunits could result in the generation of smaller fragments, and increasing the activity of ATPases could accelerate protein translocation into the proteasome, and so yield larger peptides.

1.9.1 THE PROTEASOME IN *DROSOPHILA*:

The *Drosophila* 26S proteasome has been purified (Udvardy, 1993), and shown to degrade ubiquitin-protein conjugates. The 19S particle (or μ particle) is incorporated in to the 26S proteasome in an ATP-dependent manner. Synthesis of the 19S particle is developmentally regulated, with the highest levels present in embryos.

Cloned genes encoding proteasomal subunits include an α subunit, *Dm25* (Haass *et al.*, 1990) which can be incorporated and functionally substitute for a mouse proteasomal subunit. Functional substitution is dependent on the N-terminal hydrophobic domain (Seelig *et al.*, 1993). This shows proteasomal subunits are also conserved across species.

Drosophila proteasomes can be isolated from transcriptionally inactive early embryos, so may be important for early stages of embryogenesis (Haass and Kloetzel, 1989). Proteasome accumulation in the embryo occurs in cells involved in morphogenetic movements, and the presence of proteasomes is often concomitant with the establishment of mitotic domains (Klein *et al.*, 1990). The proteasome may therefore be involved in cell specific proteolytic events required for cell proliferation and morphogenesis during early *Drosophila* development. However, it is difficult to interpret the results presented, as antibodies to the proteasome hybridise almost everywhere throughout the embryo, but are sometimes found in higher concentrations within particular cells. At later stages of embryonic development, proteasomes were detected at high concentrations in the ventral nerve cord (Klein *et al.*, 1990).

1.10 UBIQUITIN CARBOXYL-TERMINAL HYDROLASES (UCHS):

The recycling of free ubiquitin is essential in ubiquitin-dependent proteolysis. Several ubiquitin carboxyl-terminal hydrolases (UCHs) have been found, which specifically hydrolyse ester or amide bonds linked to Gly76 of ubiquitin (review: Hersko and Ciechanover, 1992). They are essential to release ubiquitin from the isopeptide linkage with Lys residues of the protein substrate, and to disassemble the polyubiquitin chains, at the final stage of the proteolytic process. Hydrolases may also have a function to release proteins which have been incorrectly ubiquitinated. Abnormal polyubiquitin chains may be "trimmed" by UCHs to enable recognition and binding to the 26S proteasome. As ubiquitin genes are arranged in linear polyubiquitin arrays, or fused to ribosomal proteins (see 1.4), hydrolases are required

for processing these ubiquitin precursors. Another hydrolase may be required to remove the one or more extra amino acids at the C-terminus of polyubiquitin gene products (see 1.4). Products from the interaction of high energy E1-, E2- or E3-ubiquitin thiol-esters reacting with intracellular nucleophiles could rapidly deplete free ubiquitin pools in the cell, so must be quickly cleaved by ubiquitin carboxyl-terminal hydrolases. An appropriate hydrolase may be responsible for removing ubiquitin from mono ubiquitinated proteins.

Most ubiquitin carboxyl-terminal hydrolases are thiol proteases (Wilkinson, 1994). They are encoded by two gene families. One family has relatively small gene products of 25-30kDa, and these enzymes prefer a small leaving group in the reactions they catalyse. The other gene products are approximately 100kDa and prefer a large leaving group (Wilkinson, 1994). The small enzymes are probably responsible for cleaving ubiquitin from the fusion proteins encoded by ubiquitin-coding genes.

Mammalian 30kDa enzymes UCH-L1, -L2 and -L3 have been found (Mayer and Wilkinson, 1989). UCH-L3 was found in humans (Wilkinson *et al.*, 1989), and is the major 30kDa UCH in mammals. UCH-L1 is PGP9.5, a protein present at high levels in neuronal tissue. Testes, liver, heart and kidney express high levels of UCH-L2 (Wilkinson, 1994), showing that each 30kDa enzyme may be tissue specific. Different tissues probably contain different ubiquitinated proteins. All three enzymes have an active site Cys residue, and catalytically essential His residues which probably bind metal ions. The 30kDa class of C-terminal hydrolases is conserved in evolution: *YUH1* is the yeast homologue of *UCH-L3*. Null mutants of *YUH1* are viable, possibly due to complementation within UCH enzyme families. YUH1 cleaves the isopeptide linkage between ubiquitin and small proteins (Miller *et al.*, 1989), as does UCH-L3.

Fusions of ubiquitin to larger proteins can be cleaved by larger yeast UCHs: ubiquitin specific proteases (UBP) 1, 2 and 3 (Wilkinson, 1994). DOA4 is another member of this group (Papa and Hochstrasser, 1993), and was found to function by cleaving ubiquitin chains from substrate remnants still bound to the proteasome. These proteins have recently been found to belong to the ubiquitin C-terminal hydrolase family 2 (UCH2: Falquet *et al.*, 1995). This family was defined on the basis of the Cys and His domains found in DOA4 (Papa and Hochstrasser, 1993), which are involved in de-ubiquitinating activity, and probably bind metal ions. An aspartic acid domain is also conserved (Falquet *et al.*, 1995), but its function is

unknown. The UCH2 family includes the *Drosophila* FAT FACETS (FAF) protein (see 1.10.1).

A pseudo KEKE domain was found in a UCH2 family member (Falquet *et al.*, 1995). These domains mediate non-covalent association between proteins (Realini *et al.*, 1994), and may mediate binding of UCH2 family proteins to the 26S proteasome. It seems reasonable to speculate some of these UCHs are present in the 19S regulatory particle of the 26S proteasome, and are responsible for cleaving polyubiquitin chains from partially degraded substrates. Eytan *et al.* (1993) characterised an ATP-dependent hydrolytic activity that is an integral part of the 26S proteasome complex. The enzyme cleaves isopeptide bonds, and appears to release ubiquitin in the terminal stages of degradation. Other UCHs in this family may be responsible for breakdown of the polyubiquitin chains. They may be associated with the 26S protease complex, perhaps via non-covalent interaction through pseudo KEKE domains, as the protein found containing this domain is probably isopeptidase T (Falquet *et al.*, 1995; see below).

Isopeptidase T (Hadari *et al.*, 1992) is a monomeric ubiquitin-binding protein, with two ubiquitin-binding sites, which acts on polyubiquitin chains, converting them to free ubiquitin. The role of this isopeptidase is to remove polyubiquitin chains following degradation of the protein substrate by the 26S proteasome (Hadari *et al.*, 1992). It disassembles the branched chains by a sequential exo- mechanism, requiring a free C-terminal Gly of the proximal ubiquitin moiety (Wilkinson *et al.*, 1995). This implies the UCH in the 26S proteasome complex must cleave polyubiquitin chains from the substrate before isopeptidase can act.

1.10.1 *DROSOPHILA* UCHS:

1.10.1.1 *Uch-D*:

A *Drosophila* UCH named *uch-D*, was cloned due to its enriched expression on the ventral side of the oocyte and in nurse cells during oogenesis (Zhang *et al.*, 1992). It is a member of the family of 30kDa UCHs, so may be responsible for cleaving ubiquitin from the *DUb52*, *DUb80* and *Ubip* (see 1.4.2) gene products.

Uch-D locates to 98F in the *Drosophila* genome (Zhang *et al.*, 1992). A 1.1kb *uch-D* transcript is seen at all stages of development, but is elevated in 0-4hr embryos, ovaries and testis. The high level of transcripts in the ovary and young embryo are typical of a maternal mRNA stored in the oocyte and used during

embryogenesis. A role for *uch-D* at this stage could be the release of ribosomal proteins from ubiquitin fusion proteins, for the rapid assembly of ribosomes during embryogenesis.

1.10.1.2 *Fat facets* (*faf*) :

The *fat facets* (*faf*) gene is a *Drosophila* UCH2 family ubiquitin carboxyl-terminal hydrolase (see above: Falquet *et al.*, 1995; Huang *et al.*, 1995). The FAF protein is required in cell communication pathways that negatively regulate neural determination in the developing compound eye (Fischer-Vize *et al.*, 1992). Null *faf* mutants are viable, but have abnormal eye morphology due to a misdetermination event, where cells become neurons that do not normally do so, resulting in more than the wild type eight photoreceptors in each eye facet (Fischer-Vize *et al.*, 1992). Mutant females lay embryos which never reach cellularisation, implying *faf* is also required during oogenesis. The first 392 amino acids of the FAF protein localise it to the posterior pole of oocytes, dependent on the presence of wild type *oskar* function (Fischer-Vize *et al.*, 1992), and ovary-specific functions may be due to this localisation.

The FAF protein can cleave ubiquitin from fusions to β-galactosidase in *E.coli,* (Huang *et al.*, 1995) and mutations of the active site Cys residue to Ser destroy this ability. FAF also functions as a ubiquitin carboxyl-terminal hydrolase in *Drosophila* as mutation of the Cys or either of the two essential His residues severely impairs *faf* functions in flies (Huang *et al.*, 1995).

Reducing the amount of proteasome subunit l(3)73Ai by half results in strong suppression of the *faf* mutant phenotype, but FAF does not appear to associate with the proteasome (Huang *et al.*, 1995). This suggests FAF activity antagonises proteasome function, possibly by deubiquitinating a specific regulatory protein targeted for degradation, before it reaches the proteasome.

Fat facets is a cell type specific UCH, and acts as a regulator of cell fate decision in *Drosophila melanogaster*.

1.11 PROTEINS DEGRADED BY THE UBIQUITIN SYSTEM:

The ubiquitin system is involved in the bulk degradation of short-lived and abnormal proteins. The proteasome is also involved in the bulk degradation of long-

lived proteins (Rock *et al.*, 1994), but as lack of E1 function does not appear to affect degradation of long-lived proteins (Gropper *et al.*, 1991), it may be ubiquitin-independent. This section shows some examples of proteins targeted by the ubiquitin system.

1.11.1 TRANSCRIPTION FACTORS:

1.11.1.1 MATα2 repressor:

The MATα2 repressor is a short-lived nuclear transcriptional regulator of mating type switching in *S.cerevisiae*. It has two degradation signals (see 1.5.2.2; Hochstrasser and Varshavsky, 1990), and is degraded by the ubiquitin system (Hochstrasser *et al.*, 1991). The rate of MATα2 degradation is significantly lower in a yeast *ubc4ubc5* deletion strain, and *UBC6* and *UBC7* are also involved (Chen *et al.*, 1993). UBC6 and UBC7 proteins form a complex together (see 1.7.1.6) and target MATα2 for degradation via the *Deg1* signal. UBC4 and UBC5 can also ubiquitinate MATα2, but recognise an unknown signal. As *UBC6/UBC7*-dependent degradation requires membrane association of the complex, MATα2 is probably degraded in this manner at spatially restricted sites in the cell. This may be a method for degrading abnormal MATα2 protein which has not been translocated to the nucleus.

1.11.1.2 NF-κB and IκBα:

In response to a wide variety of extracellular signals, the transcription factor NF-κB is activated, and results in a variety of transcriptional responses (Thanos and Maniatis, 1995). In non-inducing conditions, NF-κB resides in the cytoplasm in inactive complexes with IκBα. If NF-κB is in precursor form (p105), this also prevents translocation to the nucleus. IκBα is related to the C-terminal prodomain of p105, and both mask the NF-κB nuclear localisation signal. NF-κB is activated by processing of p105 and by destruction of IκBα, both of which occur via the ubiquitin-proteasome pathway (reviews: Deshaies, 1995a.; Hochstrasser, 1996).

Palombella *et al.* (1994) showed that processing of p105 is dependent on ATP, polyubiquitination and the 26S proteasome. p105 is partially degraded to active p50, which still requires degradation of IκBα for nuclear transportation. Degradation of IκBα requires signal-induced phosphorylation of two Ser residues

near its amino terminus (Chen *et al.*, 1995) by a specific IκBα kinase (Chen *et al.*, 1996). UBC4 and UBC5 are necessary for ubiquitination of IκBα, and also for IκBα kinase activity (Chen *et al.*, 1996). Multiubiquitination of an unknown factor, probably part of the IκBα kinase complex, must occur before phosphorylation and subsequent ubiquitination and degradation of IκBα (Chen *et al.*, 1996). Ubiquitinated IκBα remains associated with NF-κB until it is degraded. NF-κB can then move to the nucleus, and act as a transcription factor.

The ubiquitin-dependent control of NF-κB is interesting as during *Drosophila* development, dorsal-ventral axis specification requires the DORSAL transcription factor, which is homologous to NF-κB. DORSAL is normally maintained in an inactive state in the cytoplasm by the IκB homologue CACTUS. The TOLL signalling pathway triggers the degradation of CACTUS (Belvin *et al.*, 1995) to allow nuclear import of DORSAL. This pathway may also be regulated by phosphorylation-dependent ubiquitin-mediated proteolysis.

1.11.2 PHYTOCHROME:

The phytochrome protein exists in two interconvertible forms; the Pr form, absorbing light at 670nm (P_{670}) is converted to Pfr, P_{730}, following exposure to far red light. The photoconversion initiates processes adapting plants to photosynthetic light, and results in about a 100 fold increase in the degradation of phytochrome (Shanklin *et al.*, 1987). A concomitant increase in ubiquitin-phytochrome conjugates is seen (review: Callis, 1995). Following irradiation, phytochrome aggregates. Perhaps only aggregated phytochrome is recognised by the ubiquitin system, or a phytochrome specific E3 may also be light activated.

1.11.3 ONCOPROTEINS:

Alterations in the rate of ubiquitin-dependent proteolysis of certain proteins may play an important role in the development of certain cancers.

The concentration of the p53 tumour suppressor protein is dramatically reduced in cells infected with oncogenic human papillomaviruses (HPVs) even though normal levels of protein are synthesised (Scheffner *et al.*, 1993). Physical association of HPV-encoded E6 protein with p53 accelerates p53 degradation via ubiquitination. Ubiquitination requires human E2s *UbcH4* and *UbcH5*, which transfer ubiquitin to a cellular E6-binding protein known as E6-AP (Scheffner *et al.*,

1995). E6-AP is a hect domain E3 (see 1.8.3), and transfers ubiquitin to p53. Reduced levels of p53 may deregulate growth control and apoptosis in infected cells, resulting in malignancy.

Regulation of *c-Jun* activity is important for normal cell growth, and overexpression can lead to cellular transformation. Ubiquitin-dependent degradation maintains *c-Jun* protein at low levels in normal cells (Treier *et al.*, 1994). A 27 amino acid region known as the δ domain is necessary for *c-Jun* ubiquitination and degradation. Other proto-oncoproteins c-Fos (Staniscovski *et al.*, 1995) and c-Mos (Nishizaura *et al.*, 1993) are also degraded in a ubiquitin-dependent fashion.

1.11.4 CYCLINS:

Cyclins, several types of which exist, are proteins involved in cell cycle control in eukaryotes. They act by regulating the activity of cdc2 protein kinase. Mitotic cyclins are rapidly degraded at the end of metaphase to enable cells to exit mitosis and divide. Analysis of degradation targetting signals revealed the "destruction box" (Glotzer *et al.*, 1991; see 1.5.2.3). Cyclins are degraded by ubiquitin-dependent proteolysis (Glotzer *et al.*, 1991; Hershko *et al.*, 1991). Ubiquitination of mitotic cyclins requires a constitutively active E2, and an E3 in the 1000-1500kDa cyclosome or anaphase promoting complex (APC) which is a large multisubunit complex, active in mitotic, but not interphase cell extracts, necessary for degradation of mitotic B type cyclins and progression of the cell cycle to anaphase (Hershko *et al.*, 1994; Sudakin *et al.*, 1995; King *et al.*, 1995).

B-type cyclin degradation is mediated by *UBC9* in *S.cerevisiae* (see 1.7.1.8). Genes *CDC16*, *CDC23*, and*CSE1* were seen to be involved in this proteolysis as mutants stabilize cyclin B-βgalactosidase chimeras in yeast cells depleted of G1 cyclins (Irniger *et al.*, 1995). These genes were previously identified as being required for chromosome segregation and anaphase. CDC16, CDC23 and CDC27, another protein implicated in chromosome segregation (Lamb *et al.*, 1994), are subunits of the anaphase promoting complex. CDC16, 23 and 27 are highly conserved, and perform the same functions in humans (Tugendreich *et al.*, 1995), *S.pombe* (Samejima and Yanagida, 1994) and *Aspergillus nidulans* (Mirabito and Morris, 1993), suggesting they are part of the APC in all eukaryotes. Anti CDC27Hs antibodies immunodeplete the APC from *Xenopus* extracts (King *et al.*, 1995). Activation of cyclin degradation in mitosis is triggered by phosphorylation of the

APC. CDC16 and CDC27 show an increase in mass on entry to mitosis (King *et al.*, 1995), implying these are the phosphorylated APC subunits.

Non-degradable cyclin B blocks *S.cerevisiae* cell cycle in late anaphase (Surana *et al.*, 1993), but cells with t.s. *CDC16*, *CDC23* (Irniger *et al.*, 1995) or proteasomal (Ghislain *et al.*, 1993; Friedman and Snyder, 1994; Gordon *et al.*, 1993) gene products arrest in metaphase at the non-permissive temperature. Chromosome segregation is blocked by non-degradable cyclin in *Xenopus* extracts (Holloway *et al.*, 1993). These observations imply the APC promotes degradation of both cyclin B and a protein involved in sister chromatid cohesion (Holloway *et al.*, 1993), another role in the cell cycle for ubiquitin-dependent proteolysis.

1.11.4.1 Cyclin degradation and the *Drosophila* cell cycle:

During the first seven mitotic divisions of *Drosophila* embryogenesis, the levels of cyclin B and activity of cyclin-Bp34^{cdc2} do not oscillate (Edgar *et al.*, 1994). Cycles 8-13 show a progressive increase in the extent of cyclin degradation during mitosis, which leads to increasing fluctuations in CDC2 kinase activity. Cyclin B degradation may be spatially restricted to the nuclear areas in the early divisions, as the APC subunits localise to the centrosome and mitotic spindle (Tugendreich *et al.*, 1995; Mirabito and Morris, 1993).

1.12 FUNCTIONS OF THE UBIQUITIN PATHWAY:

Intracellular proteolysis does not just control the turnover of damaged or abnormal proteins. Ubiquitin-dependent proteolysis plays a key role in processes as diverse as embryonic development, receptor endocytosis and cell cycle control. This section attempts to review some functions which have not been previously discussed.

1.12.1 RECEPTOR MEDIATED ENDOCYTOSIS:

Many cell surface proteins are rapidly endocytosed, and recycled to the plasma membrane, or destroyed by vacuolar proteases in the vacuole or lysosomes. Plasma membrane proteins can be ubiquitinated, and this can serve as a tag for degradation (reviews: Hurtley, 1996; Hochstrasser, 1996).

Most cases of cystic fibrosis (CF) are caused by mutations interferring with the folding of the cystic fibrosis transmembrane conductance regulator (CFTR) leading to rapid degradation of CFTR molecules that have not matured beyond the E.R.. CFTR is targetted for proteasomal degradation by ubiquitination (Ward *et al.*, 1995). The cell performs quality control of its secretory and membrane proteins in the E.R., and it is here that CFTR of CF patients is polyubiquitinated and degraded (Jensen *et al.*, 1995).

Other intrinsic membrane proteins are presumably also substrates for ubiquitin-dependent degradation during their maturation within the E.R. However, Hicke and Riezman (1996) show ubiquitination of membrane proteins can also occur at the membrane, and marks the proteins for degradation in the vacuole, the yeast equivalent of the lysosome. Ligand-induced ubiquitination of α-factor receptor STE2 protein, a yeast mating pheromone receptor, leads to receptor-ligand complex endocytosis followed by vacuolar degradation. Mutant yeast cells lacking some ubiquitin conjugating enzymes cannot internalize and degrade the receptor in response to added pheromone (Hicke and Riezman, 1996). A nine residue sequence within the cytoplasmic C-terminal tail of the STE2 protein is critical for internalisation. Cells expressing STE2 without this region bind pheromone, but are not ubiquitinated, internalized or degraded efficiently. In cells with protease-deficient vacuoles, ubiquitinated STE2 accumulates in vacuoles, but cannot be degraded. Cells with defective proteasomes have no effect on degradation of the ligand-bound, ubiquitinated and internalized receptor.

Ubiquitination is probably often used as a signal for endocytosis and vacuole/lysosome targetting of receptors. Yeast STE6, uracil permease, amino acid transporter GAP1 and multidrug transporter PDR5 all appear to follow a similar degradation pathway to STE2 (Kolling and Hollenberg, 1994; Egner and Kuchler, 1996). A number of mammalian membrane proteins are also multiubiquitinated at the cell surface and degraded in the lysosome (Ciechanover, 1994).

1.12.2 NEURODEGENERATIVE DISEASE:

Dramatic increases in ubiquitin and ubiquitin-protein conjugates are observed in a wide variety of neurodegenerative disorders (review: Muller and Schwartz, 1995). These include Parkinson's disease (Manetto *et al.*, 1988), Alzheimer's disease (Mori *et al.*, 1987), amyotrophic lateral sclerosis (motor neuron disease; Bergmann, 1993), and Creutzfeldt-Jakob disease (Suenaga *et al.*, 1990). The same increases are

seen for animal models of the human dieases. One common feature found is the presence of filamentous inclusion bodies: abnormal cytoplasmic inclusions containing high levels of cytoskeletal proteins. Ubiquitin has been found covalently associated with some proteins in the inclusion bodies; ubiquitin is bound to the microtubule associated protein TAU of paired helical filaments in inclusion bodies in Alzheimers' brains (Mori *et al.*, 1987).

It is not yet clear whether the accumulation of ubiquitin represents a role for the protein in the pathology of these diseases. Ubiquitin levels may be elevated in order to degrade damaged proteins arising from progression of the disease, or may be unrelated to the disorder, and just reflect random accumulation of small cellular proteins.

1.12.3 UBIQUITIN IN DEVELOPMENT:

Ubiquitin was first discovered as a protein capable of inducing the differentiation of T-cell and B-cell lymphocytes (Goldstein *et al.*, 1975). The ubiquitin-dependent proteolytic pathway has other roles in differentiation of tissues and cell types in development. The tissue specificity of some ubiquitin pathway enzymes has previously been mentioned (see 1.6.3, 1.10). It is likely the degradation of certain sets of previously stable proteins, no longer necessary in a particular cell type, could lead to differentiation. Between different tissues, different sets of proteins will be expressed, so each cell type should have a ubiquitin system refined to recognise and degrade/control the half-lives of its particular expressed proteins.

1.12.3.1 Ubiquitin in plant development:

Ubiquitin with Lys48 mutated to Arg is an inhibitor of ubiquitin-dependent proteolysis. It was expressed in *Nicotiana tabacum* to assess the role of this pathway in plant development (Bachmair *et al.*, 1990). Expression of mutant mono- or polyubiquitin gave vascular tissue abnormalities, probably because vascular tissue requires extensive degradation of intracellular proteins during differentiation. Plants were smaller due to decreased internode length (Bachmair *et al.*, 1990), and overexpression of mutant polyubiquitin led to curled leaves with necrotic lesions. Perturbations of the ubiquitin system induced a programmed necrotic response. Pollen maturation appears to be the only part of plant development not requiring the

ubiquitin system (Worrall and Twell, 1994). In maturing pollen, most proteins are produced for storage, and are not fully processed.

The substrate specificity of the ubiquitin pathway does appear to be different in different plant tissues, as different E2s are seen expressed differentially with respect to different organs of the plant (Genschik *et al.*, 1994).

1.12.3.2 Ubiquitin in vertebrate development:

During erythroid differentiation some E2s increase in concentration, and some decrease (Haldeman *et al.*, 1995; Wefes *et al.*, 1995). This suggests a role for the ubiquitin pathway in eliminating previously stable proteins through the expression of new E2s. Erythroids lack nuclei, mitochondria and many cytoplasmic and cytoskeletal proteins. Induction of specific E2s may eliminate these proteins.

There is a role for ubiquitin-dependent proteolysis in the development of the testis. Certain E1s appear to be specifically expressed in testis, and may be spermatogenic (see 1.6.3). A rat E2 is also expressed at high levels in testis (Wing and Jain, 1995), and a UCH in fish is involved in sex differentiation (Fujiwara *et al.*, 1994). Expression of the fish UCH begins during sex transition and continues during maturation of the testis. A *C.elegans* E2 has also been found with gonad specific activity during the period of gonad differentiation (Jones *et al.*, 1995). This suggests the testis-determining role of the ubiquitin pathway is not restricted to vertebrates.

1.12.3.3 Ubiquitin in *Drosophila* development:

There are mutants of three *Drosophila* ubiquitin pathway genes: *ben* (see 1.7.3.3), *hyd* (see 1.8.4) and *faf* (see 1.10.1.2). These mutants suggest developmental roles for ubiquitin-dependent proteolysis in nervous system development, imaginal disc growth, eye development and oogenesis (see chapter 6). When other mutants are discovered, they may be found to affect different developmental events.

1.12.4 PROGRAMMED CELL DEATH (P.C.D.):

The deliberate and orderly removal of cells by programmed cell death (P.C.D.) is a common phenomenon during animal development (review: Schwartz, 1991). A cell's decision to die is equivalent to differentiation, as it requires the

activation of specific sets of genes. One putative cell death gene has been identified as the product of the polyubiquitin gene (Schwartz *et al.*, 1990). Ubiquitin presumably facilitates rapid removal of cellular proteins during degeneration.

The intersegmental muscles (ISMs) of the tobacco hawkmoth, *Manduca sexta*, form a model system for investigating P.C.D.. Intersegmental muscles are necessary for eclosion of adult moths from pupae on day 18 of the moth life cycle, and then die during the subsequent 30hr. The trigger for ISM death is a decline in insect hormone 20-hydroxy-ecdysone (20-HE). Polyubiquitin expression is upregulated during ISM programmed cell death (Schwartz *et al.*, 1990), and an E1, several E2s and an E3 are also induced in response to the fall in 20-HE (Haas *et al.*, 1995). Ubiquitin conjugates increase 10-fold at eclosion, when loss of muscle protein mass is maximum (Haas *et al.*, 1995). Enzymes for ubiquitin conjugation may represent genes necessary for programmed cell death.

An increase in 26S protease activity accompanies P.C.D. of intersegmental muscles (Jones *et al.*, 1995) and four new subunits are seen in the complex in response to the 20-HE trigger. Three of these subunits are related ATPases (Dawson *et al.*, 1995). The large increase and extensive reprogramming of 26S proteasome activity may facilitate rapid muscular proteolysis.

Programmed cell death of nurse and follicle cells occurs in *Drosophila* oogenesis, and a large number of specific cells die during embryonic development (Abrams *et al.*, 1993). The ubiquitin system may have a developmental role to play during these programmed cell deaths.

From the information presented in this chapter it is obvious protein ubiquitination is involved in many different processes. Control of protein levels by selective degradation is probably important for cell differentiation, programmed cell death and for correct spatial and temporal expression of proteins which are key developmental regulators. Enzymes of the ubiquitin pathway have been found to affect the development of the *Drosophila* eye, imaginal discs and parts of the C.N.S., so there are roles for ubiquitination in *Drosophila* development. Some specificity for substrate selection for ubiquitination resides in ubiquitin conjugating enzymes. Studies on the temporal and spatial expression of *Drosophila* E2s throughout development, and on the phenotypes of *Drosophila* mutant for E2 enzymes could be informative as to the functions of the whole ubiquitin system in the development of a multicellular organism.

CHAPTER 2

MATERIALS AND METHODS

2.1 MEDIA:

2.1.1 BACTERIAL MEDIA:

Luria Broth (L-broth): Difco Bacto tryptone, 10g; Difco Bacto yeast extract, 5g; NaCl, 5g; per litre, adjusted to pH 7.2.
Luria Agar (L-agar): As Luria broth with 15g per litre Difco agar.
BBL-top Agar: Baltimore Biological Laboratories trypticase, 10g; NaCl, 5g; Difco Agar, 6.5g; per litre.
Top agarose: Agarose, 0.6g; Luria broth, 100ml; 1M $MgSO_4$, 1ml.

Ampicillin to a final concentration of 100µg/ml was added to media immediately prior to use when required.

2.1.2 *DROSOPHILA* MEDIA:

"French" fly food: Oxoid No.3 agar, 7.5g; polenta, 55g; dried flake yeast, 55g; nipagen (150mg/ml made up in 95% ethanol), 10mls; dH_2O, 100mls.
Egg collection media: Difco Bacto Agar, 90g; dH_2O, 3l; apple juice, 1l; nipagin, 6g; sucrose, 100g.

2.1.3 YEAST MEDIA:

YPDA: 1% (w/v) Yeast extract; 2% (w/v) Bacto-peptone; 2% (w/v) glucose; 0.003% (w/v) adenine.
YMG/CAS: 0.67% (w/v) Yeast Nitrogen Base without amino acids; 2% (w/v) Casamino acids; 2% (w/v) glucose. For plates 2% (w/v) agar was added. Media was supplemented with nutrients where required.
YMGal/CAS: 0.67% (w/v) Yeast Nitrogen Base without amino acids; 2% (w/v) Casamino acids; 2% (w/v) galactose. For plates 2% (w/v) agar was added. Media was supplemented with nutrients where required.

2.2 MATERIALS:

2.2.1 SOLUTIONS:

AP buffer: 100mM NaCl; 50mM $MgCl_2$; 100mM Tris.HCl, pH9.5; 1mM levamisol; 0.1% Tween 20.

Boiling Mix: Stacking gel buffer, 1.0ml; 25% SDS, 0.8ml; β-mercaptoethanol, 0.5ml; 100% glycerol, 1.0ml; 0.05% BPB.

Denaturation buffer: 0.5M NaOH; 1.5M NaCl.

2x Freezing Mix: K_2HPO_4, 3.78g; tri-Na citrate, 0.27g; $MgSO_4.7H_2O$, 0.054g ; $(NH_4)_2SO_4$, 0.54g; KH_2PO_4, 1.08g; glycerol, 26.4g; make up to 300ml with dH_2O.

Gel buffer: Tris, 18.15g; SDS, 0.4g; pH to 8.9 with HCl; make up to 100ml with dH_2O.

Hybridisation solution: 0.5M NaP (1M Na_2HPO_4, 72ml; 1M NaH_2PO_4, 28ml); 7% SDS; 1mM EDTA pH 8.0.

Injection buffer: 5mM KCl; 0.1mM $NaPO_4$, pH 6.8.

Loading buffer: 40ml 250mM EDTA, pH 8.0; BPB, 0.1g; ficoll, 20g ; make up to 100ml with dH_2O.

Lysis buffer: 300mM Tris.HCl, pH 9.0; 100mM EDTA, pH 8.0; 0.625% SDS; 5% sucrose.

10x MOPS: 1M MOPS pH 7.0, 100ml; 3M CH_3COONa pH 7.0, 8.33ml; 0.5 M EDTA, 10ml; make up to 500ml with dH_2O.

10x Nick Translation buffer: 500mM Tris-HCl, pH 7.2; 100mM $MgSO_4$; 1mM DTT.

5x OLB: 0.05M β-mercaptoethanol; 1M HEPES pH6.6; 0.03Upd$(N)_6$; 0.26M $MgCl_2$; 0.1mM dATP, dTTP and dGTP.

10x PBS: NaCl, 80g; KCl, 20g; Na_2HPO_4, 0.2g; KH_2PO_4, 20g; make up to 1 litre with dH_2O.

40% PEG solution: 40% (w/v) PEG; T.E.; 1mM LiAc pH 7.5.

Phage buffer: KH_2PO_4, 3g; Na_2HPO_4, 7g; NaCl, 5g; 0.1M $MgSO_4$, 10ml; 10mM $CaCl_2$, 10ml; 1% gelatin, 1ml; make up to 1 litre with dH_2O.

Sample buffer: 20% glycerol; 100mM EDTA; 0.1% BPB.

Stacking gel buffer: Tris, 5.1g; SDS, 0.4g; pH to 6.7 with HCl; make up to 100ml with dH_2O.

STET: 8% sucrose; 0.5% Triton X-100; 50mM EDTA pH 8.0; 50mM TrisHCl pH 8.0; make up to 200ml with dH_2O.

20x SSC: NaCl, 175g; tri-Na Citrate, 88g; make up to 1l with dH_2O.

T4 ligase buffer: 5mM ATP; 200mM Tris pH7.6; 50mM $MgCl_2$; 50mM DTT; 500μg/ml BSA.

20x TAE: Tris Base, 96.8g; Glacial acetic acid, 22.8ml; 0.5M EDTA pH8.0, 40ml; make up to 1l with dH_2O.

20x TBE: Tris Base, 216g; Boric Acid, 110g; EDTA, 18.6g; make up to 1l with dH_2O.

T.E.: 10mM Tris; 50mM EDTA.

Tfb1: 1M CH_3COOK pH 7.5, 30ml; RbCl, 12g; $MnCl_2.4H_2O$, 9.9g; $CaCl_2.2H_2O$, 1.5g; glycerol, 150g; pH to 5.8 with 0.2M acetic acid; make up to 1l with dH_2O.

Tfb2: 0.5M MOPS pH 6.8, 20ml; RbCl, 1.2g; $CaCl_2.2H_2O$, 11g; glycerol, 150g; pH to 6.8 with KOH; make up to 1l with dH_2O.

10X TGS: Tris, 30.0g; Glycine, 144.0g; SDS, 10.0g; make up to 1l with dH_2O.

Transfer buffer: Glycine, 47.5g; Tris, 10g; make up to 3.3l with dH_2O.

10x Vogel buffer: 1M PIPES, pH 6.6; 50mM $MgCl_2$; 0.6mM β-mercaptoethanol.

Wash solution: 40mM NaP (as for hybridisation solution); 1% SDS; 1mM EDTA; make up to 1l with dH_2O.

2.2.2 ISOTOPES:

α-^{32}P-dCTP (3000 Ci/mM)

α-^{35}S-dATP (400 Ci/mM)

2.2.3 SUPPLIERS:

Enzymes - Boehringer Mannheim.

Isotopes - Amersham International.

General laboratory chemicals - Sigma; Fisons; BDH; Boehringer Mannheim.

2.2.4 *E.COLI* BACTERIAL STOCKS:

NAME	RELEVANT GENOTYPE	USE	REFERENCE
NM522	F' *lac*I^{q}Δ(*lac*Z)M15 Δ(*hsd*MS-*mcr*B)5($r_K^-m_K^-$ McrBC$^-$)	Used for making competent cells for general cloning applications.	Gough and Murray (1983)
NM646	P2 *mcr*A *mcr*B *mrr*$^+$ *hsd*R	Used to make plating cells for plating libraries.	Gift from Noreen Murray
Q358	*hsd*R($r_K^-m_K^+$) *sup*E	Strain used for growth of all phage except for plating libraries.	Raleigh *et al.* (1989)
SOLR™	Δ(*mcr*CB-*hsd*SMR-*mrr*), lac[F'*pro*AB, *lac*I^{q}ZΔM15] Su$^-$	For excision of phagemid from Lambda Zap II vector.	Stratagene Predigested Lambda Zap®II / EcoRI / CIAP cloning kit Manual.
XL-1 BLUE	Δ(*mcr*CB-*hsd*SMR-*mrr*) *sup*E44 *rec*A1, *lac* [F' *pro*AB, *ac*I^{q}ZΔM15, Tn10(tet^r)]	Strain used for general transformations and plating the Lambda Zap II library.	Bullock *et al.* (1987)

2.2.5 PHAGE:

NAME	DESCRIPTION	REFERENCE
λGEM11	Cloning vector. Arms derived from EMBL3.	Promega catalogue.
λ ZAP	λ phage allowing rapid excision of the pBluescript® phagemid for characterisation in a plasmid system.	Stratagene Predigested Lambda Zap®II / *Eco*RI / CIAP cloning kit Manual.
EXASSIST™ PHAGE	Contains an amber mutation preventing replication of the phage genome in a nonsuppressing *E.coli* strain.	Stratagene Predigested Lambda Zap®II / *Eco*RI / CIAP cloning kit Instruction Manual.
λcI857	λ phage with temperature sensitive *cI* gene product. DNA digested with *Hind*III or *Pst*I and used as size markers.	Sussman and Jacob (1962)

2.2.6 PLASMIDS:

NAME	DESCRIPTION and USE	REFERENCE
pBluescript II	General cloning vector.	Stratagene catalogue.
pBM125	*GAL1* promoter cloned into YCp50. Yeast centromere plasmid for overexpression of genes under galactose control.	Gift from M. Cooper.
pD4.6	pTZ18R containing the *Dhr6* cDNA on a 2.1kb *Eco*RI fragment .	Gift from M. Koken.
pD1	pNB40 containing ~4kb *UbcD1* cDNA.	Gift from S. Jentsch.
pD2	pNB40 containing ~3.7kb *UbcD2* cDNA.	Gift from S. Jentsch.
pD3	pNB40 containing ~4.2kb *UbcD3* cDNA.	Gift from S. Jentsch.
pGEM34B/S	*CDC34* cDNA cloned into pGEM-1.	Gift from M. Goebl. Goebl *et al.*, (1988).
pπ25.7wc	Helper plasmid used in P-element transformation.	Karess and Rubin (1984).
phs70Δ2-3wc	Helper plasmid for P-element transformation containing a P-element, lacking the germline specifically spliced intron, transcribed from the *hsp70* promoter.	Gift from J-M Dura.
pKS6111	Human *CDC34* cDNA cloned into pBluescript KS^+.	Gift from S. Plon. Plon *et al.*, (1993).
pSEY8	General yeast cloning vector, derived from 2μ plasmid.	Emr *et al.*, (1986).
pUAST	Plasmid for P-element transformation to allow GAL4-dependent transcription of a gene of choice. The polylinker is downstream of 5 optimized GAL4 binding sites.	Brand and Perrimon (1993).

2.2.7 *DROSOPHILA* STOCKS:

NAME	RELEVANT GENOTYPE	REFERENCE
BIRM-2	Contains 17 internally deleted P elements on the second chromosome.	Gift from K. Kaiser. Bingham *et al*. (1982).
C22C	Carries GAL4 transgene/SM5	Brand and Perrimon (1994).
D19	P[*lac ry*$^{+}$]98F	Zhang (1992).
E2	*Sp*/*CyO*; *Sb*P[*ry*$^{+}$Δ2-3]99B *ry*^{506}/TM6	Robertson *et al*., (1988)
Δ2-3	*w*; P[*ry*$^{+}$Δ2-3]99B/TM6B	Robertson *et al.*, (1988).
hsGAL4	Carries GAL4 transgene under control of a heat shock promoter on chromosome 3/TM3	Gift from C. O'Kane.
*Kr*5Y	Carries GAL4 transgene under control of the *Kr* enhancer.	Gift from M. Akam.
OrR	Wild type	Lindsley and Grell (1968).
ry^{506}	*rosy*$^{-}$	Clark *et al.* (1986)
2+3 Balancer	*Bc Elp*/*CyO*; *Ki*/TM6 *Ubx*	Gift from A. Jarman.
UAS*lac*Z	Carries *lac*Z transgene under control of the GAL4 upstream activator sequence.	Brand and Perrimon (1993).
w^{1118}	*white*$^{-}$	Hazelrigg *et al*. (1984).

For explanation of gene symbols see Lindsley and Zimm (1992).

2.2.8 OLIGONUCLEOTIDES:

NAME	SEQUENCE	COMMENTS
16Y	CCT TAA TCC TGA TCG TGC TCG G	"-" strand 3' oligonucleotide used in P element mutagenesis experiments to screen for insertions in *uchD*.
17Y	GGA CGC CAC TTG AAT CTA ATC CCG	"+" strand 5' oligonucleotide used in P element mutagenesis experiments to screen for insertions in *uchD*.
23Y	GGC GCA ACG CAG CTT TGT GTT TCG	"+" strand 5' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *UbcD1*.
24Y	CAC CTC CTT GAT AAG GGC TGT CCG	"-" strand 3' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *UbcD1*.
25Y	GGC TCT GCG TGG CCT CGT AAT TTG	"+" strand 5' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *UbcD2*.
26Y	CCT TCT GTA TGC GCT TGG CCG AGG	"-" strand 3' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *UbcD2* .
27Y	CGC ACG TTA GTC ACA GCA ACG CAC	"+" strand 5' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *ben.*
28Y	AGG GCT GGA CTC CAG TTG TCC TTC	"-" strand 3' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *ben.*
A26	TAA ACG CAA CGC ATT GCC CAG GCG	"+" strand 5' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *Dhr6* .
A79	TGC CAT TCG CTC CTT GCC TTC CTG	"-" strand 3' oligonucleotide used in the P element mutagenesis experiment to screen for insertions in *Dhr6* .
D61	CGG AAT TCA CGC AAC GCA TTG CCC	"+" strand 5' oligonucleotide for PCR mutagenesis of the *Dhr6* active site.
D62	GGT GGA ATA GCC TTG GAC ATA TTG C	"+" strand oligonucleotide for mutating DHR6 active site Cys to Ala.

D63	GCA ATA TGT CCA AGG CTA TTC CAC C	"-" strand oligonucleotide for mutating DHR6 active site Cys to Ala.
D64	GCC TCG AGC GAT CTA GGT AGC ATG	"-" strand 3' oligonucleotide for PCR mutagenesis of the *Dhr6* active site.
D6S2	GGT GGA ATA AGC TTG GAC ATA TTG C	"+" strand oligonucleotide for mutating DHR6 active site Cys to Ser.
D6S3	GCA ATA TGT CCA AGC TTA TTC CAC C	"-" strand oligonucleotide for mutating DHR6 active site Cys to Ser.
D31	CGG AAT TCG CAA TAA CTT GGC GC	"+" strand 5' oligonucleotide for PCR mutagenesis of the *ben* active site.
D32	GGC CGC ATT GCC CTC GAC GTG C	"+" strand oligonucleotide for mutating BEN active site Cys to Ala.
D33	GCA CGT CGA GGG CAA TGC GGC C	"-" strand oligonucleotide for mutating BEN active site Cys to Ala.
D34	GCC TCG AGT TCA TGT CAC GCA CCC	"-" strand 3' oligonucleotide for PCR mutagenesis of the *ben* active site.
D3S2	GGC CGC ATT AGC CTC GAC GTG C	"+" strand oligonucleotide for mutating BEN active site Cys to Ser.
D3S3	GCA CGT CGA GGC TAA TGC GGC C	"-" strand oligonucleotide for mutating BEN active site Cys to Ser.
E2 primer 1	ATC GCC GGT CCG CCT GAC CAG CCC	"+" strand oligonucleotide corresponding to postions 295 to 318 in *UbcD4* cDNA.
E2 primer 2	GAT GGA ACA CTG GAC GAT C	"-" strand oligonucleotide corresponding to postions 249 to 231 in *UbcD4* cDNA.
E2 primer 3	GGT GCG ATT TGT CTG GAC	"+" strand oligonucleotide corresponding to postions 433 to 450 in *UbcD4* cDNA.
E2 primer 4	TCT GTT CCT GCT TAC GGC	"+" strand oligonucleotide corresponding to postions 585 to 605 in *UbcD4* cDNA.
E2 primer 5	TAG GCC ACC ACT GCG TCC	"-" strand oligonucleotide corresponding to postions 563 to 546 in *UbcD4* cDNA.
E2 primer 6	TCA GCA TCA ATC CTT CAG	"+" strand oligonucleotide corresponding to postions 836 to 853 in *UbcD4* cDNA.
E2 primer 7	CTC TCT CCC TGT TCC CTG	"-" strand oligonucleotide corresponding to postions 134 to 117 in *UbcD4* cDNA.
E2 primer 8	GTT CAC CGT TCT CCT TTC	"-" strand oligonucleotide corresponding to postions 118 to 100 in *UbcD4* cDNA.

G58	**CG**G AAT TCC CAT CCA/C AAC/T GTA/G/T TAC	"+" strand degenerate oligonucleotide corresponding to HPNVY amino acids of CDC34.
G59	**CG**G GAT CCA/G GCG GC ATC C/GAC A/GTT A/GGC C/G/TGG C/G/TGA	"-" strand degenerate oligonucleotide corresponding to SPANDVAA amino acids of CDC34.
KS	TCG AGG TCG ACG GTA TC	pBluescript sequencing oligonucleotide.
N54	**CG**G AAT TCC AA/GT ATA/G TCC AC/GA/G CAA/T AT	"+" strand degenerate oligonucleotide corresponding to ICLDIL amino acids of DHR6.
N55	**CG**G AAT TCA/G GCA/C GGC/G/T GAG TG/TG ATT GGG	"+" strand degenerate oligoinucleotide corresponding to PNPNSPA amino acids of DHR6.
N56	**CG**G GAT CCG AC/GT ATC CA/CT TC/TA AA/GC CA/CA AA/GG TG	"-" strand degenerate oligonucleotide corresponding to EYPNKPPTV amino acids of DHR6.
P31	CGA CGG GAC CAC CTT ATG TTA TTT CAT CAT G	Oligonucleotide corresponding to 31bp inverted repeats of the P element.
rev	GGA AAC AGC TAT GAC CAT G	M13 reverse 19mer sequencing oligonucleotide.
RK1	GTA TCC AAA CAA ACC GCC	"+" strand oligonucleotide for sequencing *Dhr6* active site mutants.
RK2	TAT CCA ATG TCA GCG CCC	"+" strand oligonucleotide for sequencing *ben* active site mutants.
SK	CGC TCT AGA ACT AGT GGA TC	pBluescript sequencing oligonucleotide.
T7	GTA ATA CGA CTC ACT ATA GGG C	T7 promoter sequencing oligonucleotide.

All oligonucleotides are shown in 5' - 3' orientation. Extra nucleotides are shown in bold; restriction enzyme recognition sites are shown underlined - *Bam*HI = 5' GGATCC 3'; *Eco*RI = 5' GAATTC 3'; *Xho*I = 5' CTCGAG 3'.

2.3 METHODS:

2.3 .1 MANIPULATIONS OF BACTERIA AND PHAGE

2.3.1.1 Growth of *E.coli* Bacterial cultures:

Liquid culture of *E.coli* was performed in L-broth by inoculating with a single colony using a sterile metal loop. Cultures of 10mls or less were grown in 2oz glass bottles, those greater than 10mls were grown in conical flasks with a total capacity 5-10x that of the culture volume. Cells were shaken at 37°C for an appropriate length of time.

2.3.1.2 Storage of *E.coli* bacterial cultures:

For long term storage, 1/2 ml of overnight culture, grown in L-broth, with antibiotic if necessary, was mixed with 1/2 ml of 2x Freezing Mix (autoclaved) in sterile vials. Vials were frozen in liquid N_2, and stored at -70°C. To recover live cells, vials were thawed quickly at 37°C and the culture was streaked out on L-broth agar plates, with antibiotic when required.

Bacteria were stored as 5ml overnight cultures or on agar plates for short term storage (4-6 weeks).

2.3.1.3 Small scale preparation of plasmid DNA (boiling method miniprep): (Holmes and Quigley, 1981)

A single bacterial colony, containing a plasmid, was inoculated into 10ml of L-broth containing an antibiotic, if necessary. After overnight shaking at 37°C, 1.5ml of the culture was briefly spun down. The pellet was resuspended in 200µl of STET and 20µl of lysozyme (10mg/ml). The suspension was boiled at 100°C for 40sec, centrifuged for 10min and the resulting flocculent pellet was removed with a sterile toothpick.

The plasmid DNA was precipitated by addition of 22µl 3M NaAcetate and 200µl isopropanol, and being placed at -70°C for 15min and spun down for 10min. The pellet was rinsed with ether, dried under vacuum and resuspended in 50µl of T.E..

2.3.1.4 Large scale preparation of plasmid DNA (midi/maxi prep):

Large scale preparation of plasmid DNA was carried out by using QIAGEN Plasmid Midi/Maxi kits (QIAGEN GmbH and QIAGEN Inc.) according to the manufacturer's handbook instructions. This method is based on an alkaline lysis procedure, followed by binding to an anion-exchange resin under low salt and pH. Impurities such as RNA and protein are removed by a medium salt wash and plasmid DNA is eluted by high salt and concentrated by isopropanol precipitation.

2.3.1.5 Preparation of competent *E.coli* cells: (Hanahan, 1985)

E.coli cells were plated on to L-agar plates and grown overnight at 37°C. One colony was used to inoculate 10mls of L-broth and incubated at 37°C with agitation for approximately 2hr until cells reached an $O.D._{550}$ of 0.3. A subculture of 5ml of cells in 95ml L-broth was incubated until an $O.D._{550}$ of 0.35-0.6 was reached, then the culture was chilled on ice for 5min, aliquoted into corex tubes and chilled on ice for a further 10min.

Cells were pelleted by centrifugation at 6,000rpm for 5min in a cold Sorvall centrifuge. The supernatant was discarded and the pellet was resuspended in 10ml of Tfb1. After a 15min incubation on ice, cells were recentrifuged at 6,000rpm for 5min. Each pellet was resuspended in 1ml Tfb2 and the suspension was aliquoted into Eppendorf tubes, "snap" frozen in liquid nitrogen and stored at -70°C.

2.3.1.6 Transformation of plasmid DNA into competent cells:

Half a ligation reaction, or 0.1-10ng of plasmid DNA were added to 30-100µl of competent cells thawed slowly on ice.

After 30min on ice, cells were heat shocked in a 42°C water bath for 2min and chilled on ice for 2 min. Before placing the tubes at 37°C for 30-60min, 50-100µl of L-broth were added .

If blue/white selection was required, 100µl 100mM IPTG and 20µl 50mg/ml X-gal were added. Cells were plated on antibiotic containing medium to allow identification of plasmid containing colonies.

2.3.1.7 Preparation of plating cells for phage infection:

Cells were grown overnight at 37°C on L-plates. A single colony was used to inoculate 10ml of L-broth, and this was grown overnight at 37°C with shaking. A subculture of 100µl of the cells in 10ml of fresh L-broth with 0.2% maltose was grown for 4-6h, before being transferred to sterile plastic universals and spun for 10min at 3,000 rpm in a bench centrifuge. Pelleted cells were resuspended in 5ml 10mM $MgCl_2$ by vortexing. Plating cells could be kept at 4°C for several days.

Maltose improves the efficiency of bacteriophage adsorption as it induces the λ receptor (lamB protein) and Mg^{2+} ions play an important part in the maintenance and integrity of λ phage particles (Maniatis *et al.* 1982) and aid phage adsorption.

2.3.1.8 Plating and calculating titres of bacteriophage λ:

Prepared packaged bacteriophage λ particles in phage buffer were kept at 4°C. Serial dilutions of phage solution from 10^{-2} through to 10^{-8} were prepared in phage buffer. To infect cells, 10µl of each dilution were added to 100µl of plating cells and incubated at 37°C for 20min. The infected plating cells were then added to 3ml of BBL top agar, supplemented with 10mM Mg^{2+} ions, at 65°C. This was poured onto L-agar plates, left to set and incubated at 37°C overnight. The titre of the phage was calculated by counting the number of plaques formed: each plaque corresponds to one infecting phage particle.

When plaque lifts were to be taken, such as when bacteriophage λ libraries were plated, top agarose was used instead of BBL top agar.

2.3.1.9 Bacteriophage λ DNA from minilysates:

Approximately $5x10^7$ cells (100µl) from a fresh overnight culture of an appropriate strain of *E.coli*, and $1x10^8$ phage particles were added to 4ml L-broth and 40µl 1M $MgSO_4$ in a bijou bottle. Bottles were shaken at 37°C until lysis had occured (3-5hr) and any bacteria not lysed by λ were lysed by the addition of 100µl chloroform and vortexing. Debris was pelleted by centifugation for 10min in a bench centrifuge. To remove bacterial nucleic acid, 5µl 10mg/ml RNase and DNase were added, and the supernatant was transferred to a clean bijou. After shaking at 37°C for 30min, the phage solution was added to 4ml 20% PEG/NaCl in phage buffer in corex tubes. Tubes were left at 4°C overnight.

After centrifugation at 10,000rpm for 20min at 4°C, the supernatant was removed, and the phage pellet was resuspended in 0.5ml phage buffer. To ensure no bacteria remained present, 0.5ml chloroform was added, tubes were vortexed, and the phage solution was transferred to Eppendorf tubes and microfuged for 3min. Progressive phenol extraction, phenol/chloroform extraction, chloroform extraction, and ethanol precipitation were performed on the supernatant, before resuspension of the phage DNA in 100µl of T.E.

2.3.1.10 Preparation of large scale bacteriophage λ DNA:

Bacteriophage were harvested by scraping the top agarose from L-plates into glass universals. More phage were harvested by adding 4ml of L-broth to the scraped plate and transferring this to the universal. The universals were vortexed and spun in a cold bench centrifuge for 10min. The supernatant was pipetted into a glass bijou, 3-4 drops of chloroform were added, and the phage stock so formed could be kept at 4°C for several weeks.

Phage were titred (as described above) and $4x10^{10}$ phage particles were added to 200ml L-broth, 2ml $MgCl_2$ and 10ml of an overnight culture of Q358 cells at an $O.D._{650}$ of 0.5. Flasks of the culture were shaken vigorously for 3-4hr at 37°C before lysis began ($O.D._{650}$ reaches a plateau) and for a further 2-4hr until lysis was complete ($O.D._{650}$ of 0.5-1). To lyse any remaining cells, 0.5ml of chloroform were added, and shaking was continued for 10min.

Flasks were left to stand at room temperature for 1hr after addition of 8g NaCl and 20µl 10mg/ml DNase and RNase. Cultures were spun in a Sorvall centrifuge at 10,000rpm for 10min, then the supernatant was poured back into flasks containing 20g PEG and left overnight at 4°C to precipitate the phage.

After spinning down the phage at 10,000rpm for 10min and removal of the supernatant, 5ml of phage buffer were added to each sample, and the bottles were laid flat in a basin of ice and washed by the buffer for 2hr. Resuspended phage were spun down again at 5,000rpm for 5min.

The phage suspension was layered on top of CsCl step gradients, set up by aliquoting 2.5ml of CsCl (1.3g/ml) into Beckman Ultra-Clear tubes, and successively underlayering this with 2ml CsCl (1.5g/ml) and 1.5ml CsCl (1.7g/ml) using a pasteur pipette. These were spun for 40min at 18°C in a Sorvall

ultracentrifuge, and the phage band was removed by puncturing the tube with a needle on a syringe, and drawing off the band in about 0.8ml of liquid.

The concentrated phage suspension was dialysed against 1-2l phage buffer for 1-2hr at 4°C, then layered on a second CsCl step gradient and spun as before. The collected phage was dialysed against 2l T.E. at 4°C for 1hr, followed by 3 phenol extractions in Eppendorf tubes, and overnight dialysis against T.E., again at 4°C. Bacteriophage λ DNA was then ethanol precipitated and resuspended in 200µl T.E..

2.3.2 DNA MANIPULATION AND DETECTION

2.3.2.1 Phenol extraction of proteins from DNA:

Distilled phenol was equilibriated to pH 8, by mixing phenol with an equal volume of 1M Tris.HCl pH 8. Phases were allowed to separate, and the aqueous phase was discarded. This was repeated until phenol reached pH 8, then the Tris layer was replaced by T.E.. DNA to be extracted was mixed thoroughly with 0.4-1x the volume of phenol then spun for 2-5min. The upper aqueous phase was removed into a fresh tube, taking care not to transfer protein from the interface, and reextracted with phenol if necessary.

2.3.2.2 Ethanol precipitation of DNA:

DNA was precipitated from solution by addition of 0.1 volumes of 3M Na Acetate pH 5 and 2-2.5 volumes of absolute ethanol at room temperature. The solution was mixed thoroughly and left at -20°C overnight or -70°C for 15min. DNA was pelleted by centrifugation for 15min, the supernatant was discarded and the pellet washed with 70% ethanol and spun again for 2min. After removal of the supernatant, the pellet was dried under vacuum for 4-10min and the DNA was dissolved in T.E..

2.3.2.3 Digestion of DNA with restriction endonucleases:

All DNA restrictions were performed using Boehringer Mannheim enzymes and buffers. For all enzymes except *Sma*I, which digests at 26°C, 0.1-10µg of DNA were cut in 20-200µl of 1x the appropriate buffer for 1-12hr in a 37°C waterbath .

2.3.2.4 Agarose gel electrophoresis:

DNA was separated in 0.5-1.4% (w/v) Flowgen SEA KEM LE agarose with 0.5μg/ml ethidium bromide in 1xTBE buffer (for routine gels) or 1xTAE buffer (when cutting out bands from gels). Prior to loading, DNA samples were mixed with 0.1 volume sample buffer, and bacteriophage λcI857 DNA, cut with *Hind*III or *Pst*I was used as size markers. Electrophoresis was carried out horizontally across a p.d. of 1-10v/cm. DNA was visualised by UV trans-illumination.

2.3.2.5 Recovery of DNA from agarose gels:

After agarose gel electrophoresis in 1xTAE, the desired DNA fragment was cut out and extracted using Geneclean™ (Bio101). This involves binding of the DNA to a silica matrix formed by "glassmilk" and NaI. Supplier's instructions were followed. Fragments less than 200b.p.were extracted using Mermaid™ (Bio101) in which DNA binds to "glassfog". Again manufacturer's directions were followed.

Recovery of DNA using Geneclean and Mermaid was usually around 80%.

2.3.2.6 Ligation of cohesive termini:

Plasmid and foreign DNA to be ligated, were first digested with restriction endonucleases to yield complementary overhanging ends. Equimolar amounts of vector (0.1μg) and foreign DNA were mixed and dH_2O was added up to 7.5μl. The solution was warmed to 45°C for 5min to melt cohesive termini that had reannealed. Samples were then cooled to 0°C, and 1μl 10x T4 ligase buffer, and 1 Weiss unit T4 ligase (Boehringer Mannheim) were added. Samples were incubated at 22°C for 1-3hr, or overnight at 16°C.

2.3.2.7 Southern blotting: (Southern 1975)

After DNA fragments had been separated by gel electrophoresis, Southern blotting was used to transfer bands onto a nitrocellulose membrane. For genomic Southerns, 1-10μg of DNA was used per lane. To cleave fragments larger than 5kb., the gel was immersed in 0.2M HCl for 20min. The gel was then soaked in 0.5MNaOH, 1.5M NaCl for 30min to denature the DNA, and neutralised in 1.5MNaCl, 1M Tris pH 7.5 for 1hr.

The gel was placed on a support, on a wick of blotting paper which had both ends in a tray of 20x SSC. A nitrocellulose membrane (Hybond N, Amersham Intl.) the same size as the gel was placed on top, followed by 3 sheets of 2x SSC-soaked blotting paper, some dry pieces of blotting paper, a stack of paper towels and a weight, usually a heavy bottle.

The transfer was allowed to proceed for 8-14hr, before the membrane was rinsed in 2x SSC and either baked at 80°C for 2hr, or UV cross-linked for 2min to bind the DNA to the membrane.

2.3.2.8 Random primed radioactive labelling of DNA:

About 50ng-1µg of linear purified DNA was heat denatured by boiling and snap cooling on ice. The DNA was then either mixed with 30µCi α^{32}P-dCTP (3000mCi/mmol), 10µg BSA, 10µl 5x OLB and 1µl *E.coli* DNA polymerase I Klenow fragment, or an "oligo-labelling kit" (Pharmacia) was used following the manufacturer's instructions. Labelling reactions were left at 37°C for 2-12hr.

Radiolabelled DNA was separated from unincorporated nucleotides by Nuctrap™ push columns (Stratagene).

2.3.2.9 Hybridisation:

Nylon membranes, with bound nucleic acid, were placed in Techne hybridisation cylinders and 10-20ml of hybridisation solution, prewarmed to the temperature of the hybridisation, were added. The cylinders were put in Techne hybridisation ovens at the desired temperature (usually 65°C for Southerns) for a 1hr prehybridisation.

The ^{32}P-labelled heat denatured probe was added to the cylinders and hybridisation was carried out, at the same temperature as prehybridisation, for 4hr-overnight. Filters were washed 3 times in wash solution for 30min, usually at 5°C lower than the hybridisation temperature, before being wrapped in Saran wrap and placed in autoradiographic cassettes.

The radioactive signal was detected on X-ray film laid over the filters. Cassettes were stored at -70°C for an appropriate length of time, before developing the film in an automatic X-ray film processor.

Probes could be kept in hybridisation solution at room temperature and then reused, by boiling for 20min, and using this to replace the prehybridisation solution.

Nylon membranes could be stripped of probe DNA by boiling for 3min in 0.1% SDS. Filters could then be reprobed.

2.3.2.10 Colony hybridisation: (Benton and Davis, 1977)

To identify bacteria harbouring recombinant plasmids, colonies were transferred to 9cm nitrocellulose discs by placing filters onto the agar plates for 2min. Cells were lysed and DNA was denatured by soaking the discs in denaturation buffer for 2min. Discs were then neutralised on 0.5M Tris/1.5M NaCl, pH7 for 2min, washed in 2x SSC + 0.1% SDS for 2min, and finally washed twice, for 2min, in 2x SSC.

Filters were baked at 80°C for 2hr, before hybridisation was carried out, as described above.

2.3.2.11 Bacteriophage λ library screens:

Phage libraries were titred as described in 2.3.1.8. An appropriate volume of library (see Clark and Carbon, 1976) was then plated out onto large plates, using the required strain of plating cells and top agarose, and incubated overnight at 37°C.

The resulting plaques were screened directly by plaque hybridisation, whereby plaque DNA is transferred to nitrocellulose filters. First plates were chilled at 4°C to harden the top agarose, then circular Hybond N (Amersham) filters were placed on the plates for 2min, whilst a pattern of needle pricks were marked on the discs and plates by piercing through the agar. Filters were then placed in denaturing solution for 2min and neutralising solution for 2min, as for colony hybridisation. After 2min in 2x SSC, filters were baked at 80°C for 2hr.

Filters were hybridised and washed as described before, and Glogos autoradiogram stickers (Stratagene) were used to mark their positions in the cassette. Autoradiographs could therefore be aligned with the positions of plaques on the plates, and positives could be identified and picked.

The wide end of a pasteur pipette was used to pull out an agar plug surrounding the positive plaque. The agar was suspended in 500µl of phage buffer, a drop of chloroform was added, and phage were resuspended by vortexing. After 1-

2hr, dilutions were prepared and plated out on small agar plates. These were incubated overnight at 37°C.

Second screens were carried out by taking lifts from plates with low densities of plaques, in order to be able to pick single positives after hybridisation. Single positives were picked with the narrow end of a pasteur pipette and phage were resuspended in 200µl of phage buffer with one drop of chloroform.

DNA was made from positive plaques, as described above (2.3.1.9 and 10). When the cDNA λ ZAP library was being used, ***in vivo*** excision of the phagemid was performed as described in the Stratagene lambda ZapII/*Eco*RI/CIAP cloning kit manual.

2.3.2.12 Polymerase chain reaction (PCR):

All reactions were carried out using a Hybaid OmniGene programmable PCR machine. Primers were usually 18-24b.p. (see 2.2.8 for sequences) and provided by the Oswel DNA Service. For a 100µl reaction volume, 100pg template DNA was mixed with 20nmoles dNTPs, 100pmoles each primer, 2.5U TaqI polymerase (Gibco BRL), 10µl 10x PCR buffer, supplied by the manufacturers, (200mM Tris.HCl pH8.4; 500mM KCl), 5µl 1% W-1 and 3µl 50mM $MgCl_2$. Reactions were overlaid with 75µl mineral oil (Sigma).

A usual PCR run consisted of one cycle of denaturation at 94°C for 2min, annealing at 50°C for 1min and polymerisation at 72°C for 3min, followed by 25 cycles of 1min at 94°C, 1min at 50°C, and 3min at 72°C. The final cycle was usually 1min at 94°C, 3min at 50°C, 10min at 72°C. Conditions were changed depending on the T_m of specific primers and the type of PCR being performed.

Products from PCR amplifications were checked by agarose gel electophoresis. They were cloned, if necessary, by extracting the bands from gels, and, if primers had restriction endonuclease sites at their ends, by digesting and ligating as usual, or by making use of the fact *Taq*I polymerase adds a 3' terminal adenine to both strands. In this case pGEM-T Vector Systems (Promega) were used, following manufacturer's directions, to "TA clone" the products.

2.3.2.13 Sequencing double-stranded DNA: (Sanger *et al.*, 1977)

DNA sequencing was carried out using Sequenase-2 supplied by USB (United States Biochemicals), with α-^{35}S-dATP (Amersham 400Ci/mM). Boiling method miniprep DNA (3-5µg) was denatured in 0.2M EDTA, 0.2M NaOH for 30min at 37°C, and used for double-stranded sequencing. Standard annealing and termination reactions, as described by USB, were modified by the addition of DMSO. Annealing reactions were performed at 37°C for 30min, before chilling on ice. Labelling reactions were performed at room temperature for 5min, and termination reactions were left for 10min at 37°C.

Samples were loaded on a 6% polyacrylamide denaturing gel and run at approximately 40W, to maintain a gel temperature of 50-55°C. The gels were fixed in 10% methanol (v/v), 10% acetic acid (v/v) for 15min, then dried under vacuum for 1hr at 80°C and autoradiographed at room temperature overnight.

2.3.3 RNA PREPARATION AND ANALYSIS.

2.3.3.1 Collection of *Drosophila* developmental stages:

Embryos were collected on egg collection medium in petri dishes placed on the bottom of fly cages. Plates were spread with yeast to provide food for the flies. After allowing females to lay eggs for the appropriate length of time, plates were collected and embryos were washed off onto nylon mesh with distilled water. Embryos were then thoroughly washed with distilled water, and collected in Eppendorf tubes.

To collect larvae, apple juice agar plates from fly cages were left in big plastic containers, with lots of yeast, for 2-3 days until some third instar larvae could be seen. Larvae were washed off the plates and sides of the container with distilled water, and thoroughly rinsed.

Egg laying flies were placed in fresh food bottles for 4hr, then removed. Bottles were incubated for 5-10 days, and pupae (early and late) could then be collected by picking them off the sides with a paint brush.

Adult flies were anaesthetised (with carbon dioxide or diethyl ether), sexed, and collected in Eppendorf tubes.

All stages were "snap" frozen in liquid nitrogen and stored at -70°C until RNA was extracted.

2.3.3.2 Preparation of total RNA : (Chomczynski and Sacchi, 1987)

An RNAzol stock solution was made from 1 volume of stock solution A (23.64g guanidium thiocyanate; 1.25ml 1M NaCitrate, pH 7.0; 0.5g sodium lauryl sarcosine; made up to 50ml with DEPC-treated water, with 0.36ml of β-mercaptoethanol/50ml added before use), 0.1 volumes of 2M NaAcetate, pH 4.0, and 1 volume of water saturated phenol. For every 100mg tissue, 2ml of RNAzol stock were used.

Different developmental stages were homogenised in RNAzol at room temperature, to ensure solubilisation of RNA. Chloroform was added (100µl per 1ml of homogenate) and the tube was vigorously shaken for 15sec, then put on ice for 15min. After centrifugation at 10,000g for 15min at 4°C, the aqueous phase was transferred to a fresh tube and RNA was precipitated with an equal volume of isopropanol for 45min at -20°C.

The RNA pellet, from centrifugation at 10,000g for 15min at 4°C, was washed twice with 75% ethanol, dried under vacuum and dissolved in DEPC-treated 0.5% SDS at 65°C for 10min. This RNA was stored at -70°C and used for Northern blot analysis.

2.3.3.3 Preparation of ovary RNA:

Ovaries were dissected in 1 part 2x buffer mix (0.7M NaCl; 0.2M Tris.HCl, pH8.0; 0.02M EDTA; 4% SDS), 1 part 14M urea, and frozen in liquid nitrogen. Ovaries could then be stored at -70°C, or homogenized directly in more buffer.

Several phenol/chloroform extractions were performed, then the nucleic acid was ethanol precipitated with NaAcetate at -70°C for 1hr. After centrifugation, the RNA and DNA pellet was washed in 70% ethanol, dried and resuspended in 30-50µl DEPC-treated water.

RNA was precipitated overnight at 4°C,with 3 volumes of 3M LiCl, then spun down, washed with 70% ethanol, dried and resuspended in 10µl DEPC-treated water.

2.3.3.4 Northern blotting:

Approximately 10µg of total RNA samples were added to sample preparation buffer (62.5µl deionised foramide; 12.5µl 10x MOPS buffer; 20.0µl formaldehyde;

5.0µl 0.5mg/ml ethidium bromide per 10 samples) and heated to 65°C for 5min, before cooling on ice and addition of 2.5µl loading buffer. These samples were loaded on a denaturing gel (1.4g agarose; 87ml dH_2O; 10ml 10x MOPS: 2.98ml formaldehyde) and run in 1x MOPS, 2.2% formaldehyde at 25V overnight.

Gels were blotted for 8hr in 20x SSC, in the same way as Southern blots, but using a positively charged nylon membrane (Boehringer Mannheim). DNA probes were labelled as described in 2.3.2.8. Prehybridisation and hybridisation were carried out as described in 2.3.2.9, but at 55°C and with 10% dextran sulphate and 50µg/ml ssDNA in the hybridisation solution. Washes were performed at 50°C, followed by autoradiography.

2.3.4 *IN SITU* DETECTION OF DNA AND RNA.

2.3.4.1 Preparation of DIG-labelled DNA probes:

Probes labelled with DIG were synthesised by random primer labelling of DNA, either using the Boehringer Mannheim DIG DNA Labelling and Detection Kit, following instructions exactly, or by the method described below.

Approximately 100ng of DNA was mixed with 10µl of 10mg/ml $pd(N)_6$, and DEPC-treated water up to 15µl. After boiling for 5min and quick chilling on ice, 2µl 10x Vogel buffer, 2µl dNTP mix (Boehringer Mannheim DIG DNA labelling kit), and 1µl Klenow DNA polymerase (Boehringer Mannheim) were added. This was incubated overnight at 15°C, then 1µl more Klenow was added and the reaction was continued for 4hr at room temperature. To stop the reaction, 2µl 0.5M EDTA were added, and the tube was heated to 65°C for 10min. To precipitate the DNA, 80µl DEPC-treated water, 5µl 10mg/ml tRNA, 8µl 5M LiCl and 300µl ethanol were added. Tubes were left on ice for 30min, centrifuged for 20min, and the resultant DNA pellet was washed with 70% ethanol, and dried.

DIG-labelled DNA probes were resuspended in the appropriate hybridisation solution, and stored at -20°C.

The concentration of labelled DNA was determined by comparison to control DNA in the Boehringer kit, using the method described in the instruction leaflet. This method was also used when detecting DNA on Southern blots using DIG-labelled probes.

2.3.4.2 Preparation of Biotin-labelled DNA probes:

Double stranded purified linear DNA was labelled with biotin by nick translation. In this process, free 3' hydroxyl ends (nicks) are created by DNase I, then DNA polymerase I catalyzes the addition of a labelled nucleotide to the 3'-hydroxyl terminus of the nick. At the same time 5'-3' exonuclease activity of this enzyme removes the nucleotide from the 5' phosphoryl terminus of the nick, so the nick is shifted along one nucleotide at a time in a 3' direction.

The following components were mixed on ice:- 300μM dATP, 3.3μl; 300μM dCTP, 3.3μl; 300μM dGTP, 3.3μl; 3.3nmoles Biotin-16-dUTP (Boehringer Mannheim); 10x Nick Translation Buffer, 5μl; DNA polymerase I/DNase I mix (Promega), 5μl; 1-2μg DNA; dH_2O up to 50μl. Nick translation took place at 15°C for 1-2hr, before 5μl of 1.25M EDTA, pH 8.0 was added to stop the reaction.

After nick translation, the DNA solution was spun in Sephadex G50 columns to remove unincorporated nucleotides.

2.3.4.3 RNA *in situs* of whole mount embryos: (Tautz and Pfeifle, 1989)

Embryo fixation

Embryos were collected as in 2.3.3.1 and dechorionated in 50% household bleach (containing less than 5% sodium hypochlorite) for 3min. Dechorionated embryos were fixed by vigorous shaking for 15min in 1 volume 4% formaldehyde in 1x PBS, 1 volume heptane, in a scintillation vial. As much of the lower aqueous phase as possible was removed, and 8ml of methanol were added. To remove the vitelline membrane, embryos were shaken and vortexed vigorously for 10sec and, after they had settled, were collected into an Eppendorf tube and washed 2-3 times with methanol to remove any remaining heptane. Methanol was replaced with ethanol, and the fixed embryos were stored at -20°C for several weeks.

Hybridisation

Fixed embryos were rinsed in 50% ethanol, 50% xylene, then soaked in 100% xylene for several hours. After rinsing again in 50% ethanol, 50% xylene and several rinses in 100% ethanol, embryos were sequentially washed in methanol, and 50% PBT (1x PBS; 0.1% Tween 20) plus 5% formaldehyde. Postfixation was carried out for 20min in PBT plus 5% formaldehyde. Embryos were then rinsed 3 times in PBT, and incubated for 3-5min with 50μg/ml proteinase K in PBT at 37°C. Rinsing twice with 2mg/ml glycine in PBT stopped the proteinase digesting further,

and this was followed by 2 rinses in PBT, another post fixation step of 20min in PBT plus 5% formaldehyde, and 5 rinses in PBT.

Washes in 50% hybridisation solution (50% deionised formamide; 5x SSC; 100µg/ml boiled ssDNA; 100µg/ml tRNA; 50µg/ml heparin; 0.1% Tween 20), 50% PBT, then 100% hybridisation solution, preceded prehybridisation in hybridisation solution at 48°C for 1-2hr. Hybridisation was carried out overnight at 48°C in 100µl of solution containing 0.1µg/ml heat denatured DIG-labelled probe. The tube was mixed thoroughly several times during the reaction, and spun briefly the next morning to remove probe from the sides.

To remove background hybridisation of the probe, 20min washes in 1ml of the following solutions were carried out at 48°C:- hybridisation solution, 50% hybridisation solution plus 50% PBT, 5 washes of PBT.

An anti-DIG AP antibody (Boehringer Mannheim DIG-labelling and detection kit) was diluted 1:5 in PBT and preabsorbed over at least an equal volume of fixed embryos at 4°C for several hours. Preabsorbed antibody was diluted to a final concentration of 1:2000 in PBT, and probed embryos were incubated in this solution, on a rotating wheel for 1-2hr, before being washed four times in PBT (here 1x PBS, 0.1% BSA, 0.2% triton).

The next two washes were in AP buffer. The substrates for alkaline phosphatase (4.5µl NBT, 3.5µl X-phosphate (Boehringer Mannheim)) were added to the second wash to form the colour reaction. This was continued in the dark for 5min to several hours as the colour developed. The reaction was stopped by rinsing several times in PBT.

To dehydrate the embryos, PBT was serially exchanged with 70% , 95% and 100% ethanol. Several washes of 100% ethanol were carried out, including a final wash of 20min. Embryos were then stored at 4°C under ethanol before placing them in a watch glass, and replacing the ethanol with xylene. The xylene was changed 3-4 times, before embryos were mounted on slides in DPX mountant (BDH).

2.3.4.4 *In situ* hybridisation to polytene chromosomes:

Chromosome squashes

In order to get big larvae, 8-10 female flies were allowed to lay embryos in a bottle of fly food supplemented with dry yeast. Only larvae close to pupal formation were used.

Larvae were dissected in 100µl fix (1:2:3 lactic acid: water: glacial acetic acid) on a concave slide. The mouth hooks and rear were held with forceps, the head was pulled off, and salivary glands were separated from the carcass. Lateral fat was dissected off the glands, and they were sucked up in 7µl fix and put on a clean slide. A siliconized coverslip was placed on top.

To squash the glands, the slides were tapped with forceps. Chromosomes were viewed under 10x phase contrast to check they had been adequately spread out. Chromosomes were flattened by pressing hard with my thumb. Slides were left overnight at room temperature to fix the chromosomes, then plunged into liquid nitrogen. After breathing on the slides, coverslips were flipped off with a razor blade. Slides were then immersed in dry-ice-cold 95% ethanol for 5min, transferred to room temperature 100% ethanol for 5min, then air dried.

Hybridisation

Slides were incubated at 65°C for 30min in 2x SSC, transferred to 70% ethanol at room temperature for 2x 10min, then further dehydrated in 95% ethanol for 10min. After air drying, slides were boiled in Tris base/$MgCl_2$ (1.02g $MgCl_2$; 1.58g Tris Base; made up to 1l with dH_2O) for 3min to denature DNA. Slides were washed three times in 2x SSC for 5min then soaked in 70% ethanol for 2x 5min, 95% ethanol for 5min and air dried.

A 20µl drop of hybridisation solution (2x SSC; 10% Dextran sulphate; 50% deionised formamide; 2µg/ml denatured salmon sperm DNA) containing 20ng heat-denatured Biotin-labelled DNA probe was added to each slide, on top of the area of the squashes. Cover slips were laid on top, and hybridisation was carried out overnight at 37°C in a sealed box containing moist benchcoat.

After hybridisation, slides were washed as follows:- 2x 10min in 2x SSC at 37°C, 2x 10min in room temperature 2xSSC, 5min in PBS. Biotin can be detected by avidin which binds essentially irreversibly to up to four molecules of biotin at a time. Avidin was first allowed to bind to POD-linked biotin (Vector Laboratories) for 5min at room temperature in 4% nuclease free BSA and 500mM Tris, pH 7.6, before addition of 20µl of this solution to the slides. Binding of avidin to the biotinylated probes took place at 37°C in a sealed box containing moist benchcoat, for 1hr 30min.

The POD-linked biotin:avidin complex was detected using the DAB substrate (Vector Laboratories, made up as described in the manufacturer's instructions) which yields a red-brown stain in the presence of peroxidase. Before addition of 20µl of DAB to each slide, slides were washed 3x in room temperature PBS. The DAB

colour reaction was left to develop overnight at 37°C in a sealed box containing moist benchcoat. Slides were washed for 5min in PBS, then counterstained for 5min in Giesma staining solution (5% Giemsa in 50mM Na_2HPO_4/NaH_2PO_4, pH 7.2), and washed again in distilled water for 5min.

Slides were washed in xylene to make them permanent, and squashes were mounted in DPX mountant (BDH). Chromosomes were examined and scored under high power bright field and phase contrast microscopy.

2.3.5 PROTEIN EXTRACTION AND DETECTION.

All protein work was performed by Dr P. zur Lage.

2.3.5.1 Extraction of proteins from *Drosophila* and SDS PAGE:

To extract protein, flies were homogenised in boiling mix. Samples were boiled for 10min over a 100°C water bath before loading on an acrylamide gel made from 10% acrylamide in gel buffer for the resolving gel, and 3.75% acrylamide in stacking gel buffer for the stacking gel. Approximately 110µg of protein were loaded per well for Western blot analysis. Gels were run at 200V in 1X TGS.

2.3.5.2 Preparation of anti-DHR6 antibodies:

In order to raise antibodies to DHR6, a soluble GST fusion protein was injected into New Zealand rabbits. After a pre-immune sample had been taken, 0.2ml of a solution containing 100µg of DHR6 fusion protein, in a 1:1 ratio with Freund's complete adjuvant, was injected at each of 4 sites. After 10-14 days a 5ml test bleed was taken. A second series of injections was performed 4-6 weeks after the first injections using a 1:1 ratio of protein to Freund's incomplete adjuvant. A test bleed was taken after 10-14 days. This process was repeated for a third set of injections. Blood samples were centrifuged and serum was removed and allowed to clot. Supernatant was frozen in aliquots at -70°C, and this was diluted 1/1,000 in block for use as the primary antibody in Western blots.

2.3.5.3 Western Blotting:

Western blots were performed in a tank of transfer buffer using PVDF membrane (Millipore) according to the manufacturer's directions. Protein was

detected using antibodies and the Boehringer Mannheim Chemiluminescence Blotting Substrate (POD), again according to the manufacturer's instructions. The primary antibody binds to protein and is detected by a POD-linked secondary antibody. This reacts with the substrate luminol (Boehringer Mannheim), in the presence of H_2O_2, to produce light which is detected on X-ray film.

2.3.6 *DROSOPHILA* METHODS.

2.3.6.1 Preparation of genomic DNA:

No. of flies	1	10	50
Solution A (µl)	100	200	500
Solution B (µl)	14	28	70
Isopropanol (µl)	50	100	250
T.E. (µl)	10	20	100

Table 1. Amount of solutions used for making DNA from different numbers of flies.

1-50 flies were put in an Eppendorf tube at -70°C for 5min, then homogenised in solution A (0.1M Tris.HCl, pH9.0; 0.1M EDTA, pH8.0; 1% SDS; see Table 1. for amount added per fly). After incubation at 70°C for 30min, solution B (8M KAcetate) was added and tubes were left on ice for 30min.

To obtain a clear supernatant, samples were centrifuged twice at 4°C for 15min. Isopropanol was added, and DNA left to precipitate for 10min at -70°C. After a 5min centrifugation, the pellet was washed in 70% ethanol, dried under vacuum and resuspended in T.E..

2.3.6.2 DNA extraction from single flies: (Ashburner, 1989)

Single flies were homogenised in Eppendorf tubes in 50µl of homogenisation buffer (10mM Tris.HCl, pH 7.4; 60mM NaCl; 10mM EDTA, pH 8.0; 0.15mM Spermine/Spermidine; 5% sucrose). To lyse cells, 50µl of lysis buffer were added, and tubes were incubated at 70°C for 15min. After addition of 15µl 8M KAcetate and 30min incubation on ice, the homogenate was centrifuged at 4°C for 10min and the supernatant was removed to a fresh tube.

The supernatant was phenol/chloroform extracted, ethanol precipitated, and resultant DNA pellets were dissolved in 20µl T.E. plus 50µg/ml RNase A.

2.3.6.3 DNA extraction from embryos:

Embryos were collected as in 2.3.3.1., then placed in 50-100µl of homogenisation buffer (10mM Tris.HCl, pH 7.4; 60mM NaCl; 10mM EDTA; 0.15mM Spermine/Spermidine; 0.5% Triton) in an Eppendorf tube. After homogenisation, the volume of buffer was made up to 500µl and 1% w/v SDS was added. Tubes were incubated for 2-3h at 37°C after the addition of 200µg/ml proteinase K. Two phenol/chloroform extractions, and one chloroform extraction were performed, and the DNA was ethanol precipitated and resuspended in 200µl of T.E..

2.3.6.4 Establishment of transgenic lines by P element transformation:

(Rubin and Spradling, 1982)

DNA for transformation was prepared as described in 2.3.1.4, but DNA was resuspended in deionised water, not T.E. as this may kill embryos. Injection buffer containing 300ng/µl test plasmid and 100ng/µl helper plasmid (phsp70Δ2-3wc) was backloaded into the injection needle. Embryos from w^{1118} flies were collected over 30min intervals, dechorionated in 50% bleach, and washed onto a gridded filter. After lining up embryos in the same orientation alongside a grid line, they were transferred to a coverslip with glue solution (from dissolving sticky tape glue in heptane) along one edge, and dessicated in a petri dish containing silica gel for approximately 6min.

Embryos were covered with halocarbon oil, and DNA was injected into the posterior pole of preblastoderm embryos, whilst older embryos were killed by running the needle gently through them. After incubation at 18°C for 36-60hr, larvae were collected as they emerged and were left to develop at 25°C in vials of fly food. Adults were mated with w^{1118} flies, and their progeny was examined for coloured eyed flies.

Transformants were crossed to w^{1118} flies to produce males and females with the same insertion. Homozygous lines were then establised by sibling matings.

2.3.6.5 Staining embryos for β-galactosidase activity:

Embryos were collected from crossing GAL4 lines to UAS*lac*Z flies. They were washed with PBT, then dechorionated in 50% bleach. After a further wash for 2-5min in PBT, embryos were blotted dry and fixed for 7min in n-heptane saturated with PBS/2.5% glutaraldehyde. Embryos were washed with PBT for approximately 2hr until they sank to the bottom of the vial, and were washed for another 2hr, with several changes of PBT, to remove all traces of heptane.

The staining solution (10mM PO_4 buffer, pH7.2; 150mM NaCl; 1mM $MgCl_2$; 3mM $K_4[FeII(CN)_6]3H_2O$; 3mM $K_3[FeIII(CN)_6]$; 0.3% triton) was heated to 65°C until it went cloudy, and 1/50 volume of 10% X-gal in DMSO was added. Embryos were placed in staining solution and stained at 37°C in a moist chamber overnight.

After washing with PBT, embryos were mounted in 50% PBS/50% glycerol, and viewed under a microsope.

2.3.7 YEAST METHODS.

2.3.7.1 Yeast transformation:

This method was performed by Dr M. Cooper. The yeast strain to be transformed was used to inoculate 5ml of YPDA, and the culture was grown overnight to 1-2x10^7 cells/ml. Cells were then diluted in fresh YPDA to 2x10^6 cells/ml, and regrown to 1x10^7 cells/ml. After centrifugation at 3000rpm for 5min cells were resuspended in 1ml dH_2O, recentrifuged and washed once more in dH_2O, once in 100mM LiAc pH 7.5 in T.E., and finally resuspended in 100mM LiAc pH7.5 in T.E.

Aliquots of 50µl of the yeast cell suspension were mixed with 1 µg of DNA, 50µg denatured salmon sperm DNA, and 300µl of 40% PEG solution. The mixture was vortexed gently and incubated at 30°C for 30min on a rotating wheel. After heat shocking at 42°C for 15min, cells were pelleted by brief centrifugation, and resuspended in 500µl of T.E. Cells were diluted appropriately and plated onto selective medium. Plates were incubated at appropriate temperatures until the appearance of colonies.

2.3.7.2 Replica plating:

Individual colonies were picked from plates of transformants, and diluted in 120µl dH_2O in microtitre plate wells. After resuspension, a sterile nail block was used to plate a drop of each yeast colony suspension on appropriate medium. This was repeated for up to 20 plates.

2.3.8 COMPUTER PROGRAMS:

2.3.8.1 GCG programs:

Various programs from the Wisconsin Package GCG version 8.1 were used to analyse DNA and protein sequences, and to compare them to homologous sequences present in DNA and protein databases.

2.3.8.2 Phosphoimager software:

Scans from phosphoimager cassettes were analysed using Molecular Dynamics Image Quant™ software. The Volume Integration program was used to compare the relative intensity of bands on radioactive filters after exposure to a phosphoimager cassette.

2.3.8.3 Dendrogram construction and analysis:

The CLUSTALW program (Thompson *et al.*, 1994) was used to carry out an automatic multiple alignment of all E2 sequences present in the SWISSPROT protein sequence database and the UBCD4 sequence. This was then analysed using programs in the PHYLIP phylogeny inference package (Kuhner and Felsenstein, 1994).

Phylogenetic inference consists of two steps: (1.) obtaining an estimate of the best tree, and (2.) checking the accuracy of the tree obtained.

Distance-based methods of phylogenetic inference can make use of PAM (accepted point mutation) matrices (Dayhoff, 1978). These quantify the relative chance of an amino acid replacement for each of the possible changes. The Weighted Least-squares method (Fitch and Margoliash, 1967) was used for this dataset, in preference to the Neighbour-Joining method because of recent simulations showing slightly higher accuracy (see citations in Swofford *et al.*, 1996). The PROTDIST

program was used to calculate PAM-based pairwise distances from the multiple alignment. These were then fed into the FITCH program to estimate the tree.

The accuracy of the tree was tested using a statistical technique called bootstrapping. This method provides the level of "bootstrap support" for each cluster in the tree. If a cluster has high "bootstrap support" (above 90%) one can be confident it is distinct from the rest of the tree (see Swofford *et al.*, 1996 for further discussion).

2.3.8.4 Statistical analysis:

The StatView® Student data analysis package (Abacus Concepts) was used to perform statistical analysis on the results of crossing UAS*Dhr6* lines to C22C and heat shock GAL4 lines (see chapter 5 and Appendices).

CHAPTER 3

THE SEARCH FOR NEW E2S: CLONING AND CHARACTERISATION OF UBCD4

3.1 INTRODUCTION:

There is a family of E2 enzymes in every eukaryotic organism so far investigated; as yet 10 have been found in *Saccharomyces cerevisiae*, and 16 in *Arabidopsis thaliana*. At the start of the project only four E2s had been discovered in *Drosophila*, so more were likely to be present, some perhaps with interesting patterns of expression and functions in development. This chapter deals with the search for more E2 enzymes in *Drosophila*, and the characterisation of the new gene discovered.

3.2 RESULTS:

3.2.1 THE SEARCH FOR THE *DROSOPHILA CDC34* HOMOLOGUE a brief summary):

3.2.1.1 Introduction:

A likely candidate for a *Drosophila* E2 with a role in development, is the *CDC34* (Goebl *et al.*, 1988) homologue, with probable roles in normal cell cycles and the rapid cell cycles characteristic of early embryonic development. As this is an essential gene in yeast (Goebl *et al.*, 1988), and the human homologue has been found, and is also essential (Plon *et al.*, 1993), this gene should be present in *Drosophila*.

3.2.1.2 Screening Libraries Using *Dhr6*:

CDC34 is most similar to *RAD6* (Goebl *et al.*, 1988) of all other yeast E2s, and domains of each protein can functionally substitute for one another (Kolman *et al.*, 1992; Silver *et al.*, 1992). *RAD6* is 50% identical to *Dhr6* at the DNA level, so it is likely *Dhr6* is similar to the *Drosophila CDC34* homologue. Therefore a 471bp *Pvu*II/*Eco*RI fragment (figure 3.1) of *Dhr6* cDNA (Koken *et al.*, 1991a.), covering the regions of greatest homology between *RAD6* and *CDC34,* was ^{32}P-labelled and used to probe genomic Southerns of OregonR (OrR) DNA (figure 3.1B) and to screen an OrR embryo λ GEM11 genomic library. In each digest, this probe hybridised strongly to fragments corresponding in size to those expected for *Dhr6* and weakly to other fragments, which could not be from *Dhr6* but could be from the *CDC34* homologue (figure 3.2; table 3.1). These bands could not derive from partial

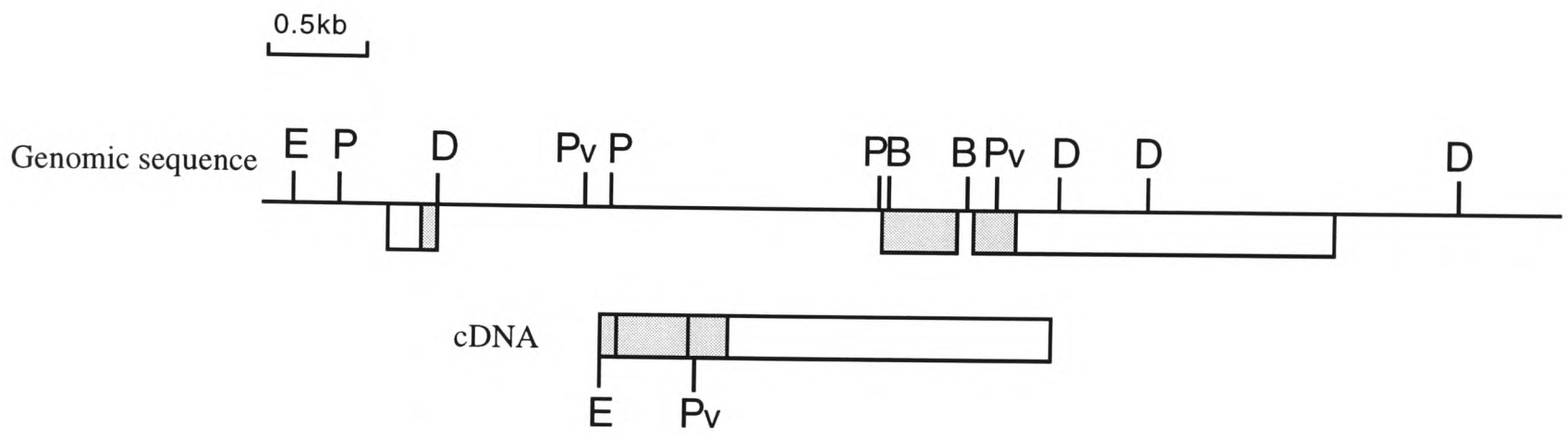


Fig. 3.1 Partial restriction map of the *Dhr6* locus (82D) and the derived cDNA, after Koken *et al.* (1991a).
Transcribed regions are shown boxed, and translated regions are dotted.
The cDNA shows the artificial *Eco*RI site by which it was cloned, and the 471bp *Eco*RI/*Pvu*II fragment used to probe genomic Southerns and the genomic library.
(E = *Eco*RI; P = *Pst*I; D = *Dra*I; Pv = *Pvu*II; B = *Bam*HI).

R.E.	*Dhr6* bands (kb)	Strong bands (kb)	Weaker bands (kb)
*Pvu*1I	1.54	1.55	2.29
*Pvu*1I/*Pst*I	0.43	0.38	0.73
*Pst*I	2.68	2.48	1.01
*Dra*I/*Pst*I	0.64	0.59	0.36
*Dra*I	2.36	2.37	3.39
*Bam*HI	0.29 3.00 3.43	0.32 3.02 3.33	3.16

Table 3.1 Size of bands expected for *Dhr6*, compared to size of bands obtained from genomic Southern of figure 3.2.

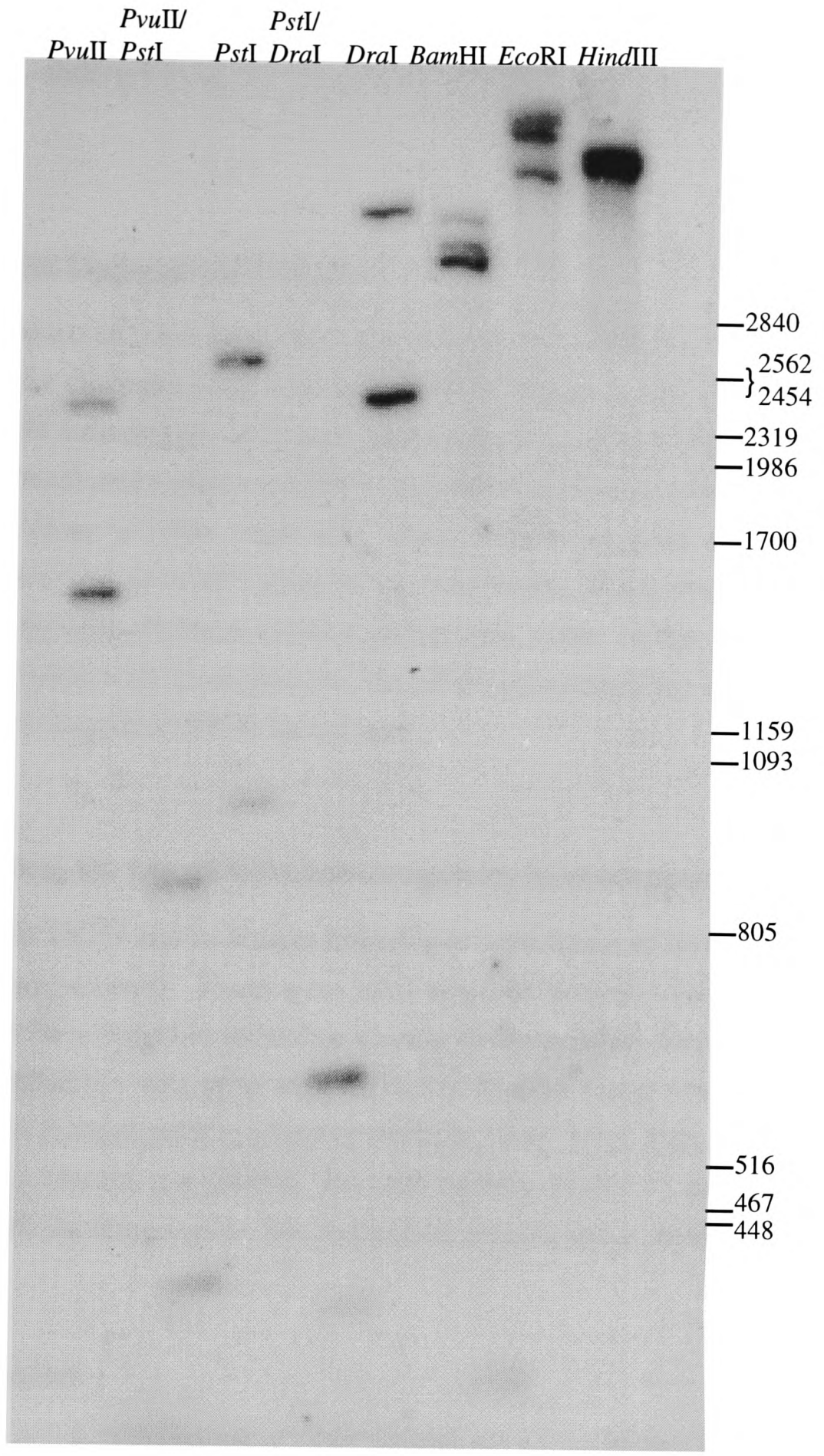


Fig. 3.2 Genomic Southern of OrR fly DNA, probed with 471bp *Eco*RI/*Pvu*II *Dhr6* fragment (Dr P. zur Lage).
Approximately 4µg DNA were loaded per lane.
Bacteriophage lambda DNA digested with *Pst*I was used as a size marker

digests as some were smaller than the bands corresponding to *Dhr6*. The five positive plaques obtained from the library screen were, however, all found to be clones of *Dhr6*.

3.2.1.3 PCR with Degenerate Primers:

One sequence of five consecutive amino acids, and another of 7/8 in *CDC34* are identical to the corresponding residues in *RAD6* (figure 3.3) and are similar to those in the *RAD6* homologues of *Schizosaccharomyces pombe* (*rhp6*), *Drosophila melanogaster* (*Dhr6*) and humans (*HHR6A* and *HHR6B*; Koken *et al.*, 1991b), but are different to those in other yeast E2s. These sequences were used to design degenerate primers G58 and G59 (Materials and Methods), taking *Drosophila* codon usage into consideration. Products of the expected size, about 180bp, were produced by PCR amplification with these primers, but after sequencing they were not found to come from a *Drosophila CDC34* homologue.

3.2.1.4 Searching for the *CDC34* homologue by homologous probing:

Clones of *CDC34* and its human homologue were received from Dr M. Goebl and Dr S. Plon respectively. These were used to probe genomic Southerns, as the human gene had been found to hybridise weakly to *Drosophila* DNA (Plon *et al.*, 1993). No hybridisation was seen with *CDC34*, despite using varying levels of stringency for the hybridisations, whereas multiple bands were seen for the human homologue probe (results not shown). An OrR embryo λ Gem11 genomic library was screened with the human gene, but no positive plaques were obtained.

3.2.1.5 Discussion:

The *Drosophila* homologue of *CDC34* was not found by any of the methods described above. As it performs an essential function in the cell cycle, and human *CDC34* hybridises to *Drosophila* genomic DNA, this enzyme should be present in *Drosophila*. It seems unlikely the *Drosophila* gene would have evolved so much as to be undetectable by these searches, which rely on homology, as human and yeast *CDC34* homologues still show 64% similarity, 36% identity at the amino acid level. Designing degenerate PCR primers to other regions of similarity between these two proteins may enable the *Drosophila* homologue to be found.

```
        1                                                        50
DHR6    ...MSTPARR RLMRDFKRLQ EDPPTGVS.. .GAPTDNNIM IWN.AVIFGP
HHR6B   ...MSTPARR RLMRDFKRLQ EDPPVGVS.. .GAPSENNIM QWN.AVIFGP
RHP6    ...MSTTARR RLMRDFKRMQ QDPPAGVS.. .ASPVSDNVM LWN.AVIIGP
UBC2    ...MSTPARR RLMRDFKRMK EDAPPGVS.. .ASPLPDNVM VWN.AMIIGP
CDC34   MSSRKSTASS LLLRQYRELT DPKKAIPSFH IELEDDSNIF TWNIGVMVLN

UBC4    .....MSSSK RIAKELSDLE RDPPTSCS.. .AGPVGDDLY HWQ.ASIMGP


        51                                                      100
DHR6    HDTPFEDGTF KLTIEFTEEY PNKPPTVRFV SKVFhpnvyA DGGICLDILQ
HHR6B   EGTPFEDGTF KLVIEFSEEY PNKPPTVRFL SKMFhpnvyA DGSICLDILQ
RHP6    ADTPFEDGTF KLVLSFDEQY PNKPPLVKFV STMFhpnvyA NGELCLDILQ
UBC2    ADTPYEDGTF RLLLEFDEEY PNKPPHVKFL SEMFhpnvyA NGEICLDILQ
CDC34   EDSIYHGGFF KAQMRFPEDF PFSPPQFRFT PAIYhpnvyR DGRLCISILH

UBC4    ADSPYAGGVF FLSIHFPTDY PFKPPKISFT TKIYhpninA NGNICLDILK


        101                                                     150
DHR6    NR........ ....WSPRYD VSAILTSIQS LLSDPNPNsp anstaaQLYK
HHR6B   NR........ ....WSPTYD VSSILTSIQS LLDEPNPNsp ansqaaQLYQ
RHP6    NR........ ....WSPTYD VAAILTSIQR LLNDPNNAsp anaeaaQLHR
UBC2    NR........ ....WTPTYD VASILTSIQS LFNDPNPAsp anveaaTLFK
CDC34   QSGDPMTDEP DAETWSPVQT VESVLISIVS LLEDPNINsp anvdaaVDYR

UBC4    DQ........ ....WSPALT LSKVLLSICS LLTDANPDdp lvpeiaHIYK


        151
DHR6    ENRREYEKRV KACVEQSFID
HHR6B   ENKREYEKRV SAIVEQSWND
RHP6    ENKKEYVRRV RKTVEDSWES
UBC2    DHKSQYVKRV KETVEKSWED
CDC34   KNPEQYKQRV KMEVERSKQD

UBC4    TDRPKYEATA REWTKKYAV.
```

Fig. 3.3 Alignment of the CDC34 amino acid sequence to that of RAD6 (UBC2) and its homologues, to show regions (lower case and underlined) used to design degenerate primers G58 and G59. UBC4 sequence is shown for comparison at these regions.
(DHR6 = a *Drosophila* RAD6 homologue; HHR6B = a human RAD6 homologue; RHP6 = an *S.pombe* RAD6 homologue).

3.2.2 THE CLONING OF *UBCD4*:

3.2.2.1 Introduction:

As the *Drosophila CDC34* homologue had not been found by any of the methods described above, I carried out a broader search for any E2s. All E2s show at least 35% homology within their UBC domains (Jentsch, 1992) so primers designed to regions of homology common to all E2s would be expected to amplify PCR products from many genes.

3.2.2.2 Degenerate primer PCRs gave products from putative new E2s:

A comparison of all known *Drosophila* E2 amino acid sequences (figure 3.4) showed regions of identity, which were then compared at the DNA level. Degenerate oligonucleotides were designed for regions containing stretches of amino acid identity (figure 3.4), taking into account *Drosophila* codon usage and that of each E2. The oligonucleotides were designed with an *Eco*RI site (coding strand primers) or a *Bam*HI site (non-coding strand primer) at their 5' ends, so that products derived from polymerase chain reaction amplifications using these as primers, could be cloned in a particular orientation. Two nucleotides (CG) were added at the start of each oligonucleotide, as restriction enzymes do not cut well if the recognition site is right at the end of the DNA fragment. The extra nucleotides result in restoration of >90% cleavage (New England Biolabs catalogue, 1990-91 p.132). The primers were called N54, N55 and N56 (see Materials and Methods for sequences) and correspond to ICLDIL, PNPNSPA and EYPNKPPTV amino acid regions of *Dhr6* respectively.

Polymerase chain reaction amplifications were set up using N54 and N56 primers, and N55 and N56 primers, with usual temperature cycles (Materials and Methods) and OrR adult DNA as the template. If no introns were present in the genomic sequence to which the primers bind, then the fragments amplified by N54 and N56, and N55 and N56 would be expected to be about 92bp and 180bp respectively. Figure 3.5 shows the results of these PCR amplifications. All bands are larger than expected, but the same bands were produced when changes were made in the PCR temperature cycles, or to the concentrations of components in the reactions. As degenerate primers were used, multiple bands were expected, but only one band of approximately 340bp was produced from the N54, N56 PCR (figure 3.5). An intron is present in *Dhr6* within the region that both sets of primers were expected to amplify, but the sites of introns in *UbcD1*, *UbcD2* and *bendless* are unknown as

```
        51                                                        100
UBCD1   .......... .......... .......... .....MALKR INKELQDLGR
UBCD2   RGSNGNGGAS GRNAGGGDEP RKEAKTTPRI SRALGTSAKR IQKELAEITL
  BEN   .......... .......... .......... ...MSSLPRR IIKETQRLMQ
 DHR6   .......... .......... .......... ..MSTPARRR LMRDFKRLQE

        101                                                       150
UBCD1   DPPAQCSAGP VGDDLFHWQA TIMGPPDSPY QGGVFFLTIH FPTdypfkpp
UBCD2   DPPPNCSAGP KGDNLYEWVS TILGPPGSVY EGGVFFLDIH FSPeypfkpp
  BEN   EPVPGINAIP DENNARYFHV IVTGPNDSPF EGGVFKLELF LPEdypmsap
 DHR6   DPPTGVSGAP TDNNIMIWNA VIFGPHDTPF EDGTFKLTIE FTEeypnkpp

        151                                                       200
UBCD1   kvAFTTRIYH PNINSNGSic ldilRSQWSP ALTISKVLLS ICSLLCDpnp
UBCD2   kvTFRTRIYH CNINSQGVic ldilKDNWSP ALTISKVLLS ICSLLTDcnp
  BEN   kvRFITKIYH PNIDRLGRic ldvlKDKWSP ALQIRTILLS IQALLSApnp
 DHR6   tvRFVSKVFH PNVYADGGic ldilQNRWSP RYDVSAILTS IQSLLSDpnp

        201                                  234 .
UBCD1   ddplVPEIAR IYKTDREKYN ELAREWTRKY AM..
UBCD2   adplVGSIAT QYLQNREEHD RIARLWTKRY AT..
  BEN   ddplANDVAE LWKVNEAEAI RNAREWTQKY AVED
 DHR6   nspaNSTAAQ LYKENRREYE KRVKACVEQS FID.
```

Fig. 3.4 Alignment of *Drosophila* E2 enzymes to show regions used to design degenerate primers (lower case and underlined). The active site Cys residues are shown in bold italics.
(N54 corresponds to the icldi/v region, N55 to p/cnpd/a/nd/spl/a, and N56 to d/eypf/m/nk/sp/apk/tv).

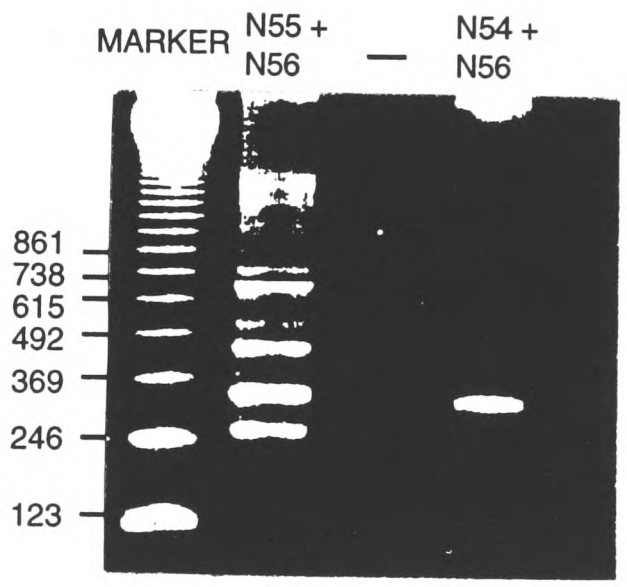


Fig.3.5 Agarose gel of degenerate primer PCR on OregonR genomic DNA. Sizes are in bp.

only cDNA has been sequenced. It is possible all *Drosophila* E2 genes have an intron at the same site as *Dhr6*, which would mean the size of bands amplified should have been larger than previously expected.

The 340bp N54, N56 PCR band was extracted from a gel, digested with *Eco*RI and *Bam*HI, ethanol precipitated and ligated into *Eco*RI/*Bam*HI digested pBluescriptII KS$^+$ (Stratagene). The five smallest fragments from the N55, N56 PCR were similarly cloned, except where the fragments did not digest well, and the strategy of "TA" cloning was employed. *Taq*I polymerase adds a 3' terminal adenine to both strands and using pGEM-T Vector Systems (Promega), the PCR products were cloned into a vector with 3'-thymidine overhangs. Fragments were then cut out of this plasmid using *Sph*I and *Sal*I sites present in the vector, and directionally cloned into pBluescriptII KS$^+$. Double-stranded sequencing was performed on DNA prepared by the boiling method using SK, KS, reverse (rev.) and T7 primers which anneal to pBluescript.

3.2.2.3 Sequencing showed part of a new E2 had been cloned:

Sequencing was carried out from both ends of each of the six PCR products cloned. At least two clones for each PCR product were sequenced. The band from the N55, N56 PCR of approximately 260bp in length (figure 3.5) was found to be part of *UbcD1* (Treier *et al.*, 1992). It consisted of the expected 182bp of coding sequence, the restriction sites on the primers, and a 64bp intron (figure 3.6) found between nucleotides 304 and 305 of the coding region of the gene. Three other bands from this PCR were found to be artifacts due to the same primer annealing to each end, and the fifth product cloned was found not to be an E2 enzyme due to the lack of a cysteine codon within the sequence, necessary in this region as the active site residue.

The 340bp fragment from the other PCR was seen to have the necessary coding capacity for an E2 in this region (figure 3.7), interrupted by an intron. This fragment was purified, and used as a probe on Southern blots of OrR adult genomic DNA, and to screen genomic and cDNA libraries. The Southern blots showed one or two bands hybridising for each enzyme used (results not shown), showing this fragment does come from the *Drosophila* genome.

```
                .         .         .         .         .
PCR BAND     1 gactatcccttcaaaccccccaaggtgGCTTTTACAACGCGCATATACCA 49
               |||||||||||| |||||| ||||| ||||||||||||||||||||||||||
UbcD1      370 gactatcccttaaaccacccaaagtgGCTTTTACAACGCGCATATACCA 419
                .         .         .         .         .
PCR BAND    50 TCCAAACATCAACAGCAATGGATCGATTTGTCTCGATATATTAAGATCTC 99
               |||||||||||||||||||||||||||||||||||||||||||||||||||
UbcD1      420 TCCAAACATCAACAGCAATGGATCGATTTGTCTCGATATATTAAGATCTC 469
                .         .         .         .         .
PCR BAND   100 AGTGGTCGCCAGCATTAACTATTTCAAAAGGTGAGTTCATCTAAGTTCTA 149
               |||||||||||||||||||||||||||
UbcD1      470 AGTGGTCGCCAGCATTAACTATTTCAAAAG..................... 497
                .         .         .         .         .
PCR BAND   150 CATCGAAAATGATACTAACATGTGTTTCCATATTCTCTTTCAGTTTTATT 199
                                                        |||||||
UbcD1      498 .....................................TTTTATT 506
                .         .         .         .
PCR BAND 200 ATCAATTTGCTCTCTACTCTGTGATcccaatccaaactcccctgct
               |||||||||||||||||||||||||||||||| ||   |||  |
UbcD1    507 ATCAATTTGCTCTCTACTCTGTGATcccaatccagacgatcctctt
```

Fig. 3.6 The 245bp N55, N56 PCR product aligned with the *UbcD1* cDNA sequence to show the site of the 64bp intron. Sequences differ only at the sites of the primers, shown in lower case. Intron splice sites are shown in bold, and the putative splice branch-point of the intron is underlined.

```
                E   Y   P    F   K   P   P    K
      1  gagtatccct ttaagccgcc aaaggtgCGT AATAGCCATG TCAGACTTTG

     51  TGCAGCCTAT ACATATGCTA GCTTAGAAAT AAACAACTCT TACATGAGTA

    101  CACCTCATCT TTCAAAAATC GTATCTTATG ACTTCACTTC GTAGAAGTGA

    151  CACTTCTATG AATCCACAAT GTTAACTTGA ATTCTGAAAG GCGCAATAAG

    201  AACCTGTAAC TTGTAATTGA AACCTAAATA AATTGCAAAC TTCTTCGCAG

          V   R   F    I   T   R   I    W   H   P    N   I   S    S   V   T   G
    251  GTACGCTTCA TAACGCGCAT CTGGCATCCG AATATATCGT CGGTTACGGG

           A   I   C    L   D   I   L
    301  TGCGatttgt ctggacatac tg
```

Fig. 3.7 DNA sequence of 322bp PCR product from N54, N56 PCR. Primer regions are shown in lower case, and the putative intron region is underlined. Appropriate splice sites are found 5' and 3' of the intron, and the predicted protein translation shown above the DNA, is similar to other E2 enzymes for this region.

3.2.2.4 Genomic and cDNA sequences corresponding to the PCR product were cloned from libraries:

An OrR λ GEM11 genomic library (provided by Kim Kaiser), and an embryo CantonS cDNA library in λ ZAP (Stratagene) were titred. The ^{32}P-labelled N54, N56 PCR product, used as a probe, gave five positive plaques from the primary screen of the genomic library, and five from the cDNA library. After secondary screens this was reduced to three positives from the genomic library, and one from the cDNA library.

Phage DNA was made from minilysates of the genomic positives, and this was digested with *Xho*I, *Bam*HI and *Xho*I/*Bam*HI. These enzymes cut inserts out from the lambda arms (figure 4.7). These digests showed two positives were clones of the same genomic region as they gave the same size bands (results not shown). The third positive also gave the same bands for the *Bam*HI digest, but more smaller bands for the *Xho*I digest and the double digests, implying it also contained the same genomic region, but covered a slightly larger area. Southern blots were performed and showed an *Xho*I fragment of approximately 13kb, and a *Bam*HI fragment of approximately 11kb hybridised to the probe used for the library screens.

In order to have enough DNA to be able to clone appropriate genomic fragments, large scale λ DNA preparations of the two different positives were made. Autoradiographs of Southern blots of restriction enzyme digests showed *Sal*I digested the genomic region hybridising to the probe to a fragment of approximately 6kb in size (results not shown), which was the same for both positive clones. This fragment was subcloned into pBluescriptII KS$^+$ (Stratagene). The 6kb *Sal*I fragment was cut by *Eco*RI into fragments of 2.2kb and 4kb, both of which hybridised to the probe. These fragments were in turn cloned into pBluescriptII KS$^+$ and sequenced.

No subcloning was necessary from the cDNA library, as the λ ZAPII® vector can be used in conjunction with ExAssist™ phage, to allow excision of the pBluescript SK$^-$ phagemid containing the cDNA clone. "Packaged" pBluescript DNA is then mixed with fresh *E. coli* cells and spread on L-ampicillin plates to produce colonies. This procedure was carried out for phagemids from the cDNA positive plaque, and minipreparations of pBluescript DNA were made. *Eco*RI digests showed the cDNA clone was approximately 900bp long, and hybridised to the probe in Southern blots.

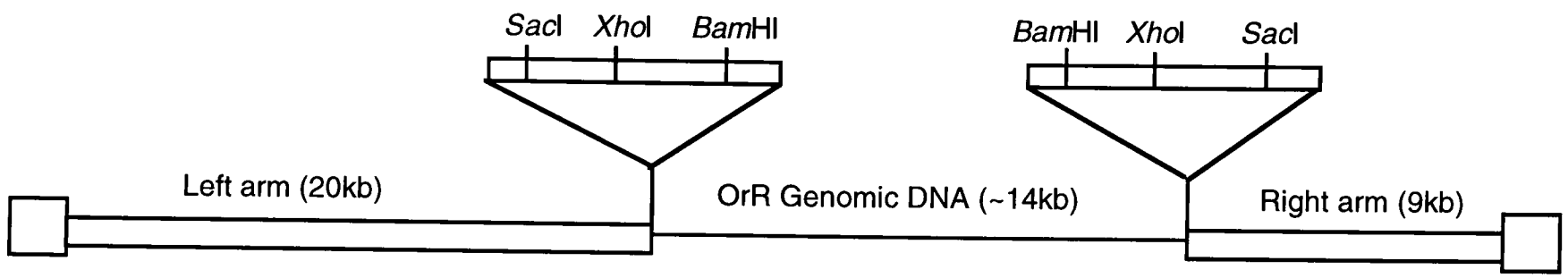


Fig. 3.8 Structural map of λGEM11 OrR genomic library (not to scale).

3.2.2.5 Genomic and cDNA Sequences of a new *Drosophila* E2 enzyme had been cloned:

The cDNA clone was sequenced completely on both strands using SK, KS, T7, and rev primers which anneal to the plasmid, and the degenerate primers N54 and N56 and E2 primers 1, 2, 4 and 5, which anneal within the insert. The E2 primers 1-7, N54, N56, T7 and SK primers were used to sequence the genomic clones. The genomic sequence was not determined completely on both strands: approximately 75% of each strand was sequenced.

The sequences of the cDNA and genomic clones align with each other, and with the PCR product of N54 and N56 primers (figure 3.9), showing that the equivalent cDNA and genomic regions have been cloned. The expected features of a gene are found in the cDNA and genomic sequences (figures 3.10 and 3.11 respectively). The 945bp cDNA has an ORF of 597bp, which is interrupted by 2 introns (figure 3.12), and codes for a protein of approximately 21.9kDa (figure 3.10). The protein was seen to be a novel *Drosophila* E2 enzyme by amino acid sequence comparison with other known E2s. It is structurally a class 2 E2 (Jentsch *et al.*, 1990), having a C-terminal extension to its UBC domain, and has a putative active site cysteine residue (by sequence comparison) at amino acid position 92.

The gene was named *UbcD4*, for *Drosophila* ubiquitin conjugating enzyme number four. The name reflects the order of identification of *Drosophila* E2 enzymes, and does not relate to homologues in other species.

The *UbcD4* cDNA sequence has been deposited in the EMBL/GenBank nucleotide sequence database, accession number X92838.

3.2.2.6 Mapping the genomic region around *UbcD4*:

OrR adult DNA was digested overnight with various restriction enzymes, a Southern blot was performed, and the filter was probed with ^{32}P-labelled *UbcD4* cDNA. The autoradiograph (figure 3.13) was used to make a partial restriction enzyme map of the genomic region surrounding the *UbcD4* gene (figure 3.14).

The fragments detected in the genomic Southern corresponded to those identified previously using the N54, N56 PCR product as a probe (data not shown). Other weaker bands could be seen in each track of the genomic Southern (figure 3.13). These bands are not due to partial digestion of DNA, and may correspond to a related gene.

```
PCR band   1 .........GAGTATCCCTTTAAGCCGCCAAAGGTGcgtaatagccatgt 41
                     |||||||| || || |||||||| ||||||||||||||||
Genomic  751 gtgcccgagACATATCCCTTCAATCCACCAAAGGTAcgtaatagccatgt 800
             |||||||||||||||||||||||||||||||
cDNA     343 gtgcccgagACATATCCCTTCAATCCACCAAAG.................

PCR band  42 cagactttgtgcagcctatacatatgctagcttagaaataaacaactctt 91
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Genomic  801 cagactttgtgcagcctatacatatgctagcttagaaataaacaactctt 850
cDNA         ..................................................

PCR band  92 acatgagtacacctcatctttcaaaaatcgtatcttatgacttcacttcg 141
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Genomic  851 acatgagtacacctcatctttcaaaaatcgtatcttatgacttcacttcg 900
cDNA         ..................................................


PCR band  142 tagaagtgacacttctatgaatccacaatgttaacttgaattctgaaagg 191
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Genomic   901 tagaagtgacacttctatgaatccacaatgttaacttgaattctgaaagg 950
cDNA          ..................................................

PCR band  192 cgcaataagaacctgtaacttgtaattgaaacctaaataaattgcaaact 241
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Genomic   951 cgcaataagaacctgtaacttgtaattgaaacctaaataaattgcaaact 1000
cDNA          ..................................................

PCR band  242 tcttcgcaggtacgcttcataacgcgcatctggcatccgaatatatcgtc 291
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Genomic  1001 tcttcgcaggtacgcttcataacgcgcatctggcatccgaatatatcgtc 1050
              | ||||||  ||||||||||||||||||||||||||||||||||||||||
cDNA          .........gcacgctttataacgcgcatctggcatccgaatatatcgtc 417

PCR band  292 ggttacgggtgcgATTTGTCTGGACATACTG..................  322
              ||||||||||||||||||  |||||||  |
Genomic  1051 ggttacgggtgcgATTTGCTTGGACATCTTAaaggacaactgggctgcag 1100
              ||||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA      418 ggttacgggtgcgATTTGCTTGGACATCTTAaaggacaactgggctgcag 467
```

Fig. 3.9 Comparison of sequences of cDNA, and genomic clones to the N54, N56 PCR amplification product sequence.
Genomic and cDNA sequences differ in two positions, probably because they were cloned from different *Drosophila melanogaster* strains.
The PCR product differs from the genomic and cDNA sequences only in the regions where the primers anneal (shown in capitals).

```
   1  caaaatccaa aattgcacgg gggggcaaca ataaaaacag aggcagaaca

  51  gaacacagca agaagagcgt ggtgaagagg agcggcggag aaaggagaac

 101  ggtgaacagg gaacagggag agagcagaaa ggagagtccg agaaacggag

                M   A   N   M   A   V   S   R   I   K   R   E   F   K
 151  gaaacatcat ggcgaacatg gcagtgtcgc ggatcaagcg ggagttcaag

       E   V   M   R   S   E   E   I   V   Q   C   S   I   K   I   E   L
 201  gaggtgatgc gcagcgagga gatcgtccag tgttccatca aaatcgaact

         V   N   D   S   W   T   E   L   R   G   E   I   A   G   P   P
 251  ggtcaatgac agttggacgg agctgcgcgg cgagatcgcc ggtccgcctg

       D   T   P   Y   E   G   G   K   F   V   L   E   I   K   V   P   E
 301  acacgcccta cgagggcggc aagttcgtcc tggagatcaa ggtgcccgag

       T   Y   P   F   N   P   P   K   A   R   F   I   T   R   I   W   H
 351  acatatccct tcaatccacc aaaggcacgc tttataacgc gcatctggca

         P   N   I   S   S   V   T   G   A   I   C   L   D   I   L   K
 401  tccgaatata tcgtcggtta cgggtgcgat ttgcttggac atcttaaagg

       D   N   W   A   A   A   M   T   L   R   T   V   L   L   S   L   Q
 451  acaactgggc tgcagcgatg acactgcgca ccgtactgct gtcgctgcag

       A   L   L   A   A   A   E   P   D   D   P   Q   D   A   V   V   A
 501  gcgctcctgg ccgccgcaga gccggacgat ccacaggacg cagtggtggc

         Y   Q   F   K   D   K   Y   D   L   F   L   L   T   A   K   H
 551  ctatcagttt aaggacaagt acgatctgtt cctgcttacg gccaagcact

       W   T   N   A   Y   A   G   G   P   H   T   F   P   D   C   D   S
 601  ggaccaacgc gtatgcgggc ggaccgcaca cctttcccga ttgtgattca

         K   I   Q   R   L   R   D   M   G   I   D   E   H   E   A   R   A
 651  aagatccaac gtctcaggga catgggcatc gacgagcatg aggcgcgcgc

           V   L   S   K   E   N   W   N   L   E   K   A   T   E   G   L
 701  cgtgctctcc aaggagaatt ggaatttgga aaaggccacc gagggcctgt

       F   S   *
 751  tcagttagct tgtcccagcc gaactagcat cagtaccatt cgcggcggtg

 801  gcagcaccaa gaacaccggc caacttcagc atcaatcctt cagaacggca

 851  atcacgctaa atgtacactc acgcaccaat atattttcgt aaatcttatc

 901  cacgaaatta cattgccctg tagttataat aaaaaaaaaa aaaaa
```

Fig. 3.10 *UbcD4* cDNA nucleotide sequence with amino acid sequence of the encoded protein. The putative active site cysteine residue is shown in bold and underlined.
cDNA was sequenced on both strands.

-202 ggaaaaacaa aaaagaaata gcgctggcaa aatagacgtg ctgagcgctg

-152 taaaaagtca gattcgtgtt gaaattggaa tattaagttt tttattttcc

-102 gtgcgctggt cgagctcgtt gcttcgacaa ttcgaaaagc gatcgtaagg

-52 agcaaccttg taggccaaca gccaggcgta atttacgcaa cgcacaacac

-2 tc**acaaaatc caaaattgca cgggggggca acaataaaaa caggggcaga**

48 **acagaacaca gcaagaagag cgtggtgaag aggagcggcg gagaaaggag**

98 **aacggtgaac agggaacagg gagagagcag aaaggagagt ccgagaaacg**

148 **gaggaaacat cATGGCGAAC ATGGCAGTGT CGCGGATCAA GCGGGAGTTC**

198 **AAGGAGGTGA TGCGCAGCGA GGAGgtgagt cccgagcacc aaggccgaag**

248 **gacactcaaa cggcacctcg gcaatgccag ctgcaccacc gcccctggtc**

298 **atggtgcagg ggggatcggg ggagggtgtc ggcgaacgtt ggtggcgttt**

348 **ttggcgcgct tatgggctcc gttttgtttg tgaaatgtaa acagtggccc**

398 **actgattgta ttgctgtaat ccctgaacag ATCGTCCAGT GTTCCATCAA**

448 **AATCGAACTG GTCAATGACA GTTGGACGGA GCTGCGCGGC GAGATCGCCG**

498 **GTCCGCCTGA CACGCCCTAC GAGGGCGGCA AGTTCGTCCT GGAGATCAAG**

548 **GTGCCCGAGA CATATCCCTT CAATCCACCA AAGgtacgta atagccatgt**

598 **cagactttgt gcagcctata catatgctag cttagaaata aacaactctt**

648 **acatgagtac acctcatctt tcaaaaatcg tatcttatga cttcacttcg**

698 **tagaagtgac acttctatga atccacaatg ttaacttgaa ttctgaaagg**

748 **cgcaataaga acctgtaact tgtaattgaa acctaaataa attgcaaact**

798 **tcttcgcagG T^{C}ACGCTTC^{T}AT AACGCGCATC TGGCATCCGA ATATATCGTC**

848 **GGTTACGGGT GCGATTTGCT TGGACATCTT AAAGGACAAC TGGGCTGCAG**

898 **CGATGACACT GCGCACCGTA CTGCTGTCGC TGCAGGCGCT CCTGGCCGCC**

948 **GCAGAGCCGG ACGATCCACA GGACGCAGTG GTGGCCTATC AGTTTAAGGA**

998 **CAAGTACGAT CTGTTCCTGC TTACGGCCAA GCACTGGACC AACGCGTATG**

1048 **CGGGCGGACC GCACACCTTT CCCGATTGTG ATTCAAAGAT CCAACGTCTC**

1098 **AGGGACATGG GCATCGACGA GCATGAGGCG CGCGCCGTGC TCTCCAAGGA**

1148 **GAATTGGAAT TTGGAAAAGG CCACCGAGGG CCTGTTCAGT tagcttgtcc**

1198 **cagccgaact agcatcagta ccattcgcgg cggtggcagc accaagaaca**

1248 **ccggccaact tcagcatcaa tccttcagaa cggcaatcac gctaaatctt**

```
1298  gtacactcac gcaccaatat attttcgtaa atcttatcca cgaaattaca

1348  ttgccctgta gttataataa ttctctgatc tgttctataa ttttattatg

1398  attaatgtgc acatctttgc cgctgggtgg gtcaccagaa ggttgagcca

1448  aaggccatgc cgcgcgcccc attaagcatg tggtgttatg taattgaacc

1498  caggttctgt ttcattaatt tacgtgcagt cgcatttact tgcaggctct

1548  tcttaaacag tactgttgtt ttccatttca agcttaaact aaaacacaaa

1598  caaaaatgat ttacaaaaca aaataaatgt gaaggcgagg atgtgataac

1648  aaatcg
```

Fig. 3.11 *UbcD4* genomic nucleotide sequence.
Transcribed sequences, according to the cDNA clone, are shown in bold, and translated regions in upper case.
-Assuming the 5' end of the cDNA is the transcriptional start site, then the underlined sequences could be the putative TATA box and CAAT box. The consensus for a TATA box is TATAAAA (7bp of A.T base pairs), surrounded by GC-rich DNA, situated approximately 25bp upstream of the transcription start. Here the putative TATA box starts at -24bp, is surrounded by GC base pairs, and consists of TAATTTA. CAAT boxes are usually found 75bp upstream of the transcription start, and have the consensus sequence GGCCAATCT, in either orientation. The putative CAAT box has the sequence CGAGAATTC and is at -78.
-Putative splice branch-point sequences are indicated by dotted lines. They match the consensus sequence of PyNPyPyPuAPy, and are positioned 18-40 nucleotides upstream of the 3' splice site, CAG.
-The presumed polyadenylation signal is doubly underlined. Polyadenylation signals are usually 11-30bp upstream of the polyadenylation site, and are highly conserved with a consensus sequence of AAUAAA. This site was chosen due to closely matching the polyadenylation signal, and being at the correct position with respect to the polyA regions found in the cDNA clone. However, mRNA studies (see chapter 4) show ~1.8kb mRNA for *UbcD4*, not ~1kb as expected from the cDNA clone. This implies a polyadenylation site matching the consensus may be present further downstream from the genomic sequence shown here.
-Superscripts show the position of base differences between cDNA and genomic sequences. The one at position 815 does not result in an amino acid change, whereas the base change at position 808 results in a Val residue being present in the genomic translation, and an Ala in the cDNA protein sequence. Both base changes are probably due to different strains of *Drosophila melanogaster* being used; genomic DNA was sequenced from Oregon R, and cDNA from CantonS.

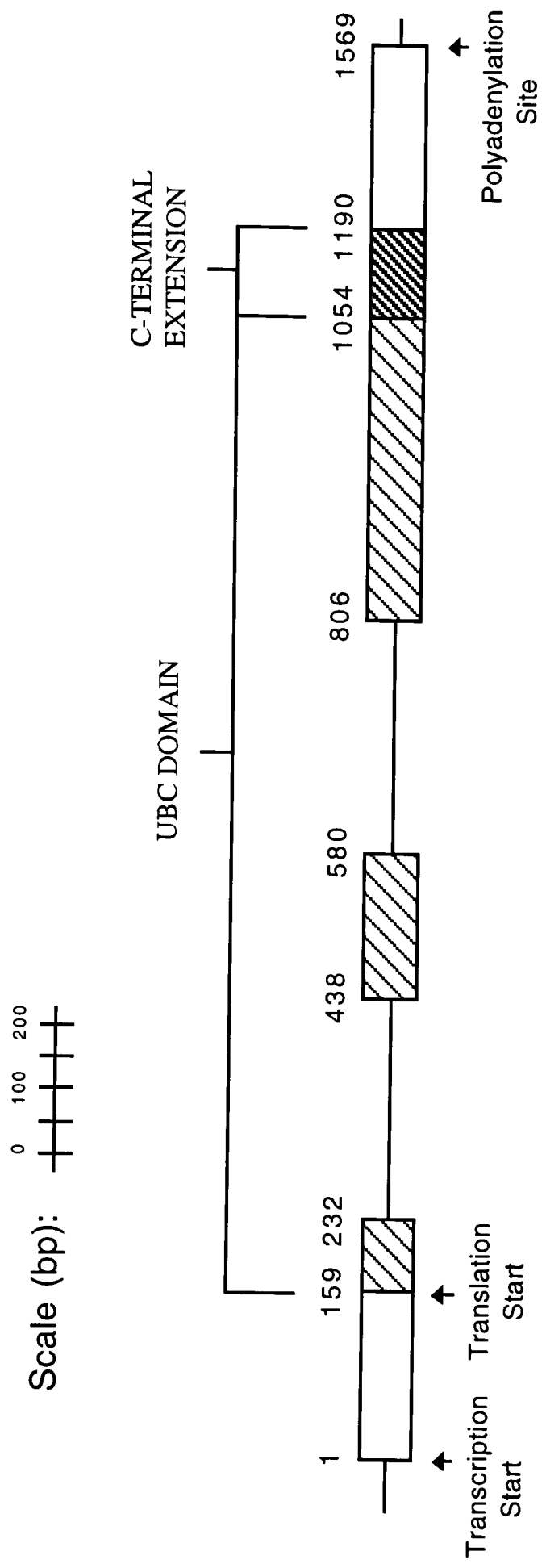


Fig. 3.12 Exon map of *UbcD4*. Translated regions are shown striped.

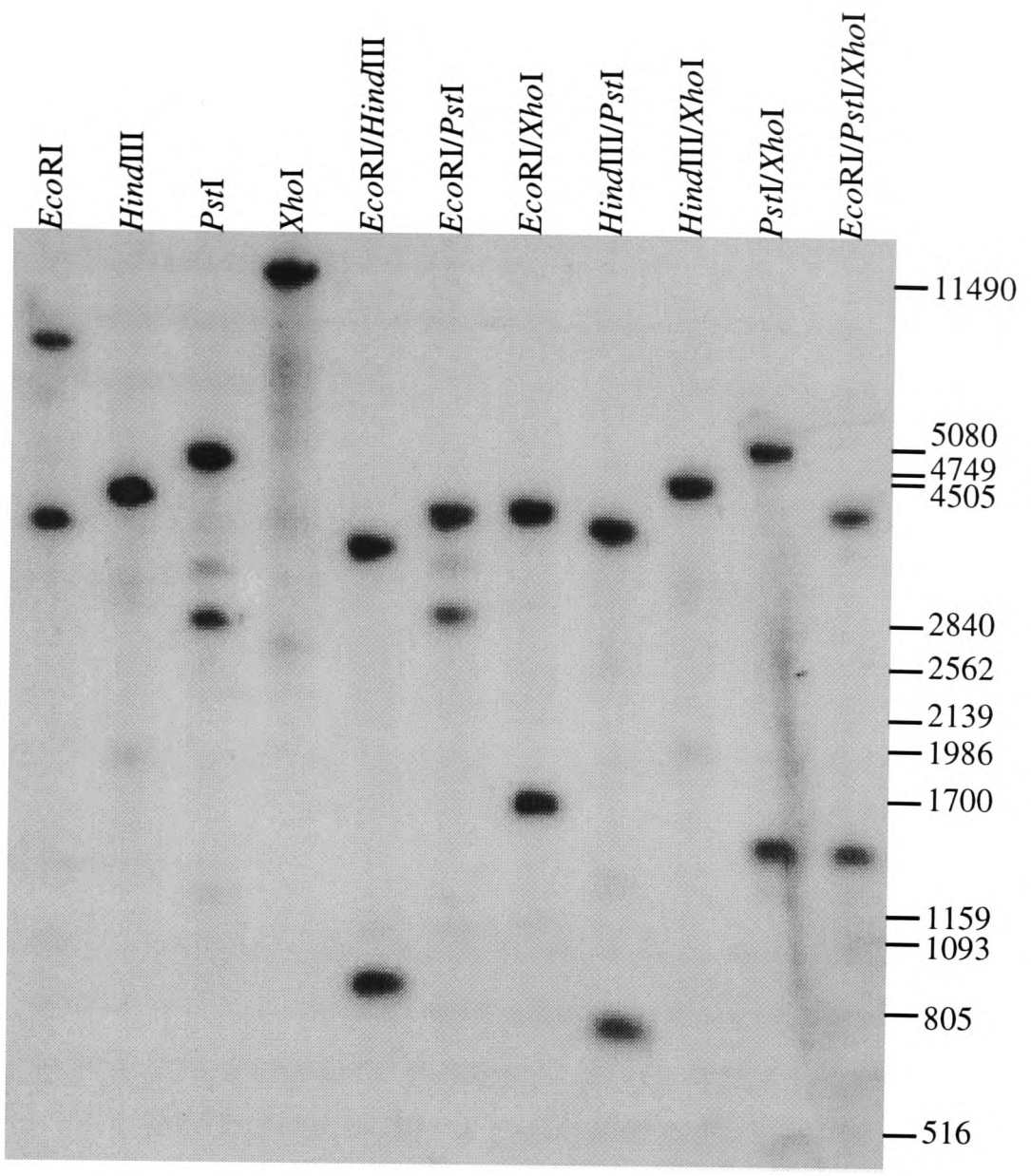


Fig. 3.13 Genomic Southern of OrR adult DNA, probed with *UbcD4* cDNA.
Approximately 4µg DNA were loaded per lane.
Bacteriophage lambda DNA digested with *PstI* was used as a size marker.

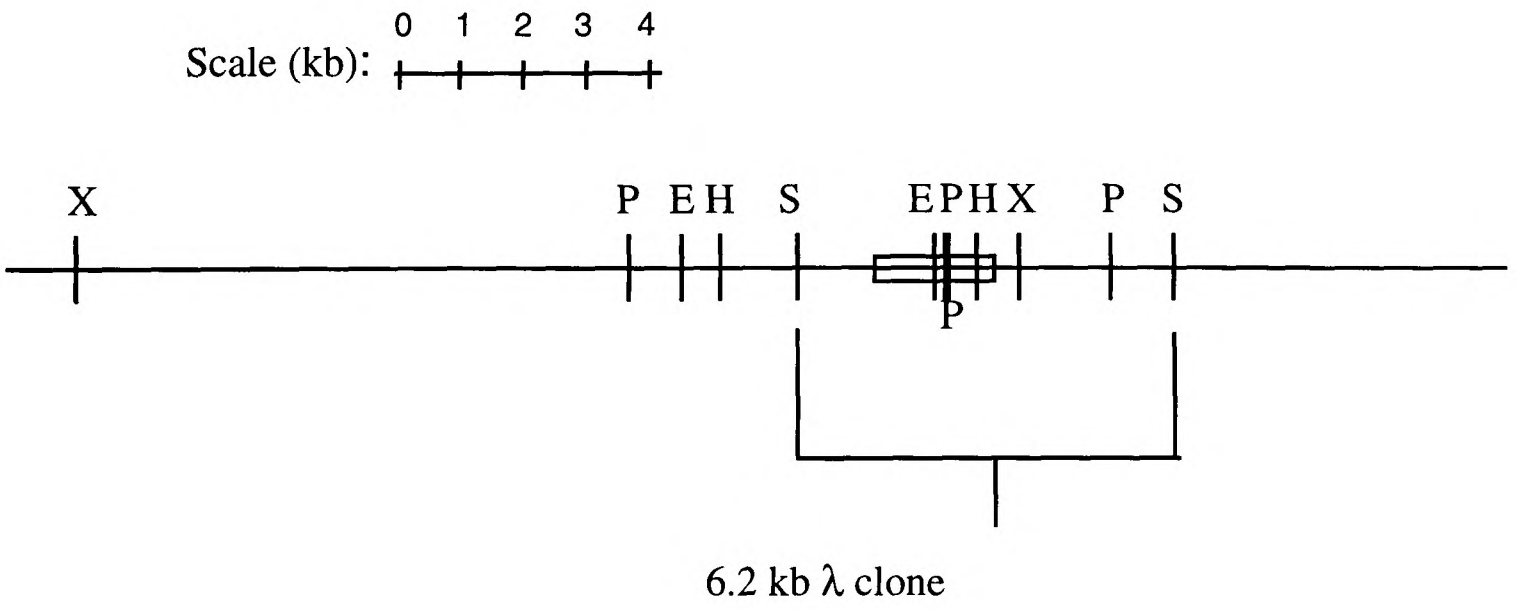


Fig. 3.14 Partial restriction enzyme map of genomic region around *UbcD4*. Boxed region shows the position of the *UbcD4* gene.
E = *Eco*RI; H = *Hind*III; P = *Pst*I; S = *Sal*I; X = *Xho*I.

3.2.2.7 The *UbcD4* gene is located at position 67C5-C11 on chromosome 3L:

In situ hybridisation to polytene chromosomes, using biotinylated*UbcD4* cDNA as a probe, was used to determine the location of *UbcD4* in the genome. The probe hybridises to position 67C5-C11 (figure 3.15) on chromosome 3L (Lefevre, 1976).

In the same way the *Duba1* gene (DNA provided by Dr P. zur Lage) was seen to localise to position 46A (figure 3.16) on chromosome 2R.

3.2.3 THE RELATIONSHIP OF *UBCD4* TO OTHER E2 ENZYMES:

3.2.3.1 Introduction:

Sequence comparisons had shown *UbcD4* was an E2 enzyme. Database searches were used to find the enzyme most related to *UbcD4*, in order to determine something about its possible roles in *Drosophila*. E2 enzymes tend to be named with respect to order of identification within a particular organism, as a result it is not obvious which enzymes are likely to be functional homologues. This question has been addressed by using sequence comparison programs to establish the phylogenetic relationships between all E2 sequences in the SwissProt database.

3.2.3.2 *UBCD4* is an E2 enzyme related to Bovine $E2_{25K}$:

The protein most similar to *UBCD4* is $E2_{25K}$ (Chen *et al.*, 1991), a bovine E2 enzyme (Figure 3.17). Chen *et al.* (1991) have shown $E2_{25K}$ is similar to UBC1 and UBC4/5 of *Saccharomyces cerevisiae* (Seufert *et al.*, 1990; Seufert and Jentsch, 1990) and UBCD4 shows approximately the same similarity to these enzymes (table 3.2). *UBC1*, *4* and *5* are essential yeast genes which together make a functionally overlapping E2 subfamily, each being able to at least partially complement mutations in the other two. This family appears to be responsible for degrading most abnormal and short-lived proteins (Johnson *et al.*, 1992; Seufert and Jentsch, 1990; Seufert *et al.*, 1990). Homologues of these genes are encoded by a multigene family in *Arabidopsis thaliana* (Girod *et al.*, 1993) and it seems probable both *UbcD4* and the $E2_{25K}$ gene are part of respective multigene families in *Drosophila melanogaster* and *Bos taurus* .

Fig. 3.15 Hybridisation of *UbcD4* cDNA to polytene chromosomes from OrR larval salivary glands.

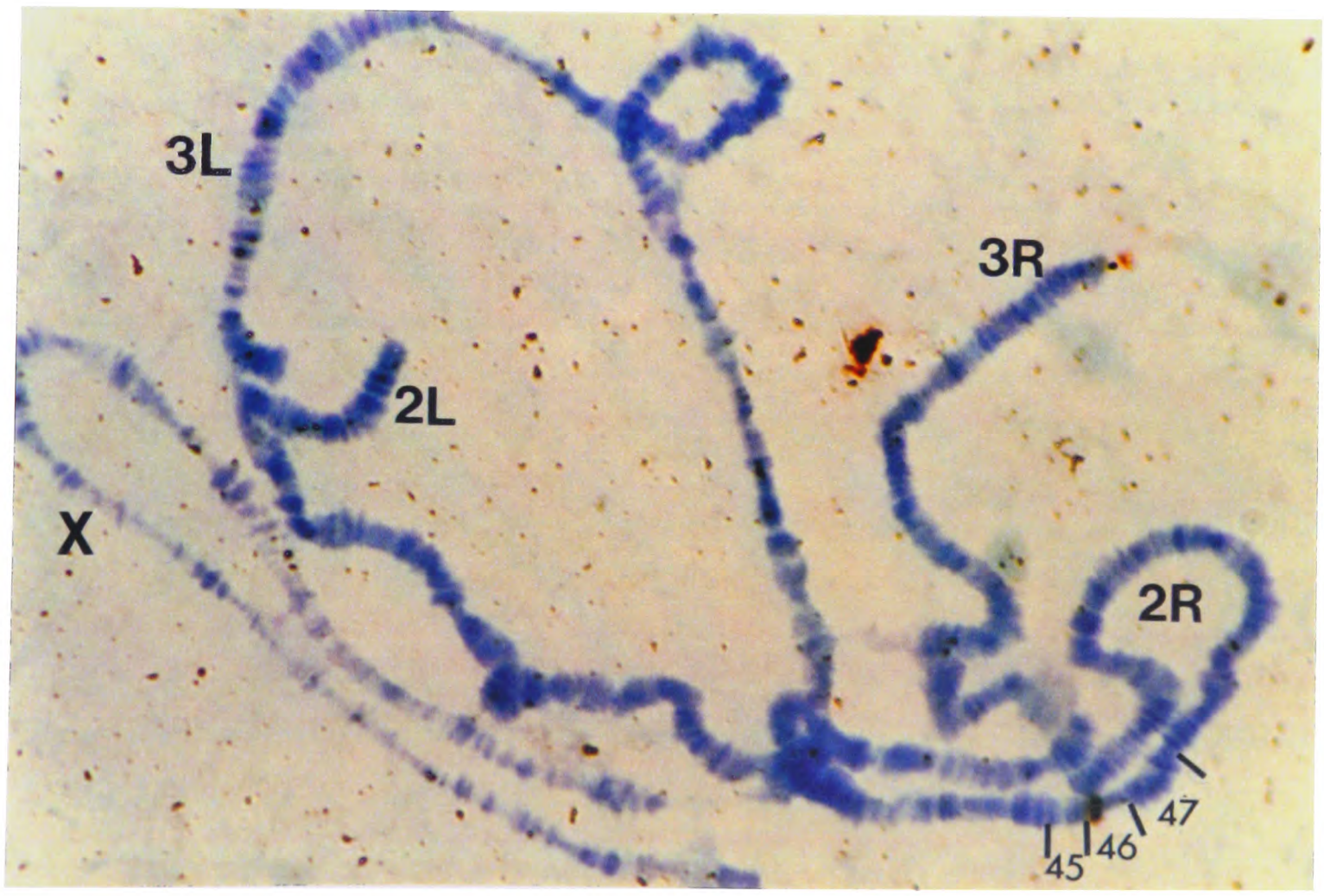


Fig. 3.16 Hybridisation of *Duba1* DNA to polytene chromosomes from OrR larval salivary glands.

```
          1                                                        50
UBCD4     MANmAVsRIK REFKEVmrSE EivqcsIKie LVndswTELR GEIAGPPDTP
E2 25K    MANiAVqRIK REFKEVlkSE EttknqIKvd LVdenfTELR GEIAGPPDTP

          51                                                      100
UBCD4     YEGGkfvLEI KvPETYPFNP PKaRFITrIW HPNISSVTGA ICLDILKDnW
E2 25K    YEGGryqLEI KiPETYPFNP PKvRFITkIW HPNISSVTGA ICLDILKDqW

          101                                                     150
UBCD4     AAAMTLRTVL LSLQALLAAA EPDDPQDAVV AyQfKdkydl FllTAkhWtn
E2 25K    AAAMTLRTVL LSLQALLAAA EPDDPQDAVV AnQyKqnpem FkqTArlWah

          151                                                     200
UBCD4     aYAGgPhtfP dcdsKIqrLr dMGiDehear avLSkenWnl EkATEgLfS.
E2 25K    vYAGaPvssP eytkKIenLc aMGfDrnavi vaLSsksWdv EtATElLlSn
```

Fig. 3.17 Comparison of amino acid sequences of UBCD4 and $E2_{25K}$, identical residues in upper case.
Overall, proteins show 81% similarity, 68% identity (to the nearest %). Their UBC domains (amino acids 1-156) are 88% similar and 76% identical at the amino acid level.

	UBCD4				$E2_{25K}$			
	TOTAL PROTEIN		UBC DOMAIN		TOTAL PROTEIN		UBC DOMAIN	
	% similar	% identical	% similar	% identical	% similar	% identical	% similar	% identical
UBC1	64	43	71	51	66	42	72	50
UBC4	57	45	57	45	59	44	59	44
UBC5	57	45	57	45	59	44	59	44

Table 3.2 Comparison of UBCD4 and $E2_{25K}$ proteins to UBC1, 4, 5, the yeast E2 enzymes for bulk degradation of proteins via the ubiquitin pathway. Values shown are to the nearest percent.

3.2.3.3 E2 enzymes can be divided into subgroups, in which UBCD4 and $E2_{25K}$ belong to the same group as UBC1:

In order to see the relationship between UBCD4 and all other E2 enzymes in the SwissProt database, their complete amino acid sequences, and those of their UBC domains were used to construct dendrograms (see Materials and Methods). The UBC domain tree is shown in figure 3.18. The dendrogram of total E2 proteins showed the same groupings, and is not shown. UBCD4, $E2_{25K}$ and UBC1 are grouped together in the dendrogram, implying that they are homologues. All three are class II E2s, having C-terminal extensions to the UBC domain, and all differ more in their C-termini than across the UBC domains (table 3.2).

A yeast specific function may be determined by the C-terminal domain of UBC1, for example UBC1 is involved in early stages of growth after spore germination (Seufert *et al.*, 1990). Other C-terminal extensions of yeast E2s have been shown to provide yeast specific functions; *Dhr6,* a class I E2, can complement all of the *rad6* mutant phenotypes bar the sporulation defect (Koken *et al.*, 1991a.) which requires the function of the C-terminal domain (Morrison *et al.*, 1988).

Analysis of figure 3.18 also reveals other groups into which E2 enzymes could be divided, and may help define other functionally overlapping E2 subfamilies. This could allow for better nomenclature; instead of enzymes being named with respect to order of discovery, they could be named by organism and E2 subgroup to which they belong.

As a lot is known about *S. cerevisiae* E2 enzymes, it would be appropriate for subgroups to refer to the yeast E2s they contain. Obvious subgroups from figure 3.14 are those containing UBC3/7, UBC9, UBC2, UBC8, UBC1 and UBC4/UBC5. A particular E2 could be named using the initials from the species name, e.g. Dm for *Drosophila melanogaster*, UBC for ubiquitin conjugating enzyme, and a number referring to the yeast E2 present in the subgroup of E2s to which the enzyme belongs. As an example, UBCD4 would be renamed DmUBC1. However, this could become complicated when two or more E2s from the same organism fall into the same group (they could be given a letter e.g. the Arabidopsis UBC9 could be known as AtUBC4a) and when species have the same initials. It would be useful to know which E2 genes are homologues from their names, and the proposed scheme for nomenclature would be possible for the E2s in figure 3.18, but genes for more E2s have been cloned, and with the discovery of many more E2s this scheme might become unworkable.

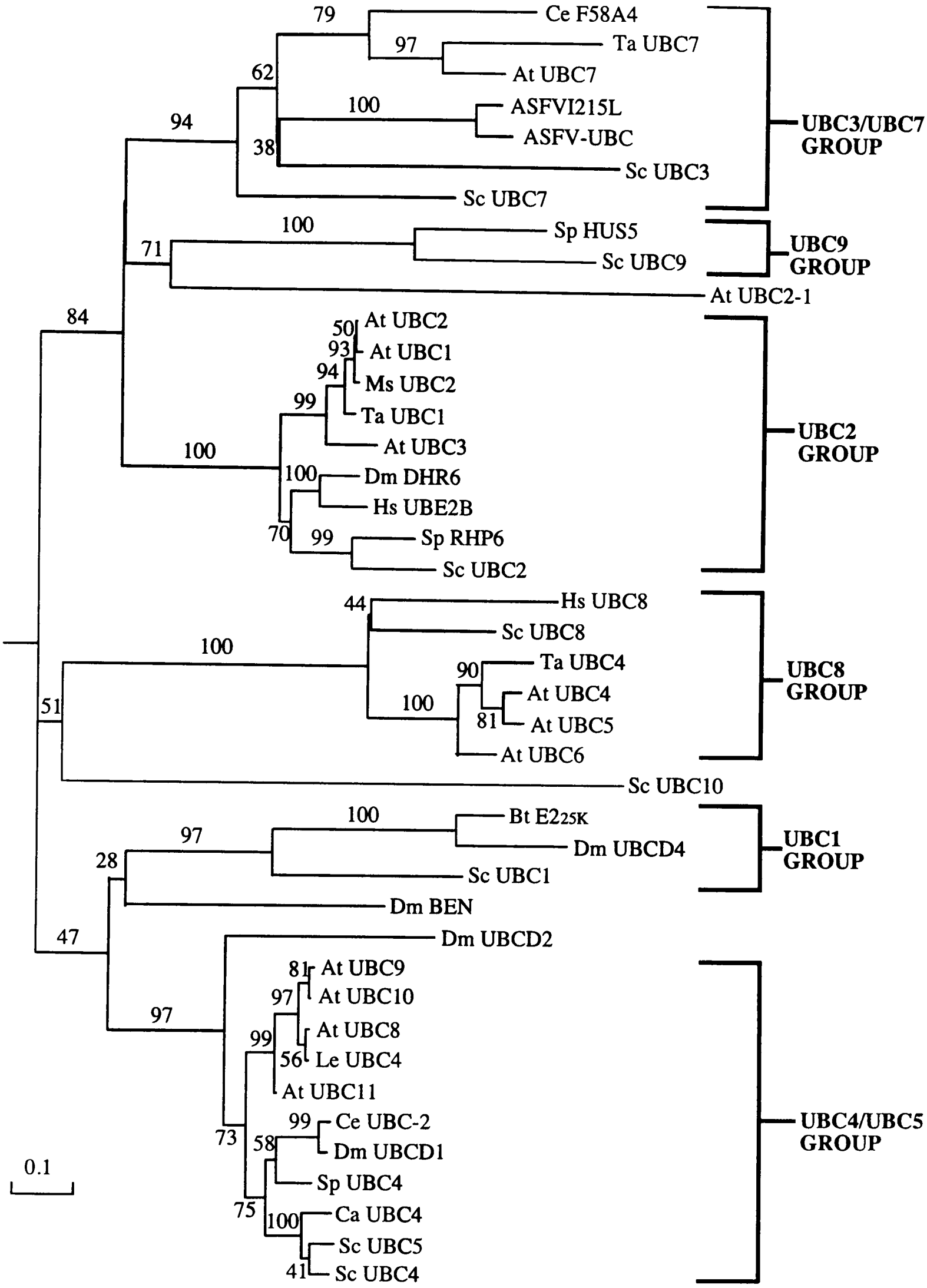


Fig. 3.18 Dendrogram of UBC domains of all E2 enzymes present in the SwissProt database. Branchpoint values show the percentage likelihood of being correct.
The scale is in substitutions per position.Yeast UBC6 does not appear in the tree as it was too distant from other E2s to be included.

At = *Arabidopsis thaliana*; ASFV = African swine fever virus; Bt = *Bos taurus*; Ca =*Candida albicans* ; Ce = *Caenorhabditis elegans*; Dm = *Drosophila melanogaster*; Hs = *Homo sapiens*; Le = *Lycopersicon esculentum*; Ms = *Medicago sativa*; Sc = *Saccharomyces cerevisiae*; Sp = *Schizosaccharomyces pombe*; Ta = *Triticum aestivum*

3.2.3.4 UBCD4 may be unable to functionally substitute for UBC1:

UBCD4 had been identified as an E2 enzyme only by sequence analysis. One functional assay for E2 activity would be to see if it could substitute for another E2 enzyme. Some *Drosophila* E2s can complement mutations in their yeast homologues:UBCD1 can functionally substitute for UBC4 (Treier *et al.*, 1992), and DHR6 can complement a *rad6* mutant for all functions except those of the RAD6 C-terminal tail (Koken *et al.*, 1991a.). The dendrograms described above imply *UbcD4* is the *Drosophila* homologue of *UBC1*. If *UbcD4* can complement mutations in any yeast E2 gene, it is likely to be *UBC1*.

The *ubc1* mutant phenotype is moderately slow growth, and markedly slow growth after germination (Seufert *et al.*, 1990). Complementation of this mutant phenotype would be difficult to determine. *S.cerevisiae* with mutations in both *UBC4* and *UBC5* cannot grow at 37°C, and this phenotype can be complemented by overexpressing *UBC1* carried on a 2μ derived plasmid (Seufert *et al.*, 1990). This suggested an alternative method for testing the functions of *UbcD4*.

UbcD4 cDNA was cloned into two different plasmids, both containing a URA3 selectable marker, under the control of the *GAL1* promoter (figure 3.19). One vector used was a 2μ-derived plasmid, the other was a low copy number centromere plasmid, with overexpression due to induction by galactose. Dr M. Cooper transformed YW023 (*ubc4*, *ubc5*, *ura*3-52) yeast cells with 1μg of DNA from each construct, and with pBM125 and pSEY8 plasmids as controls. After transformation Dr M. Cooper grew cells on YMG/CAS medium minus uracil to select for the URA3 containing plasmids, and at 23°C due to the temperature sensitivity of the strain.

Twelve colonies of each type of transformant were replica plated on YMG/CAS, YMGalRaff/CAS and YMGal/CAS, all minus uracil, at 18°C, 23°C, 30°C, 34°C, 36°C and 37°C. YMGalRaff/CAS plates were used as the strain is very slow growing, and here could use Raffinose as an additional carbon source to Galactose.

All transformants grew on all media at 18°C, 23°C, 30°C, and 34°C. At 36°C, the yeast were all slow growing on all media, and at 37°C no growth was seen for any transformant on any medium. This could be for a number of reasons, as it is not known if UBCD4 was being overexpressed. UBCD4 may not have been expressed at all.

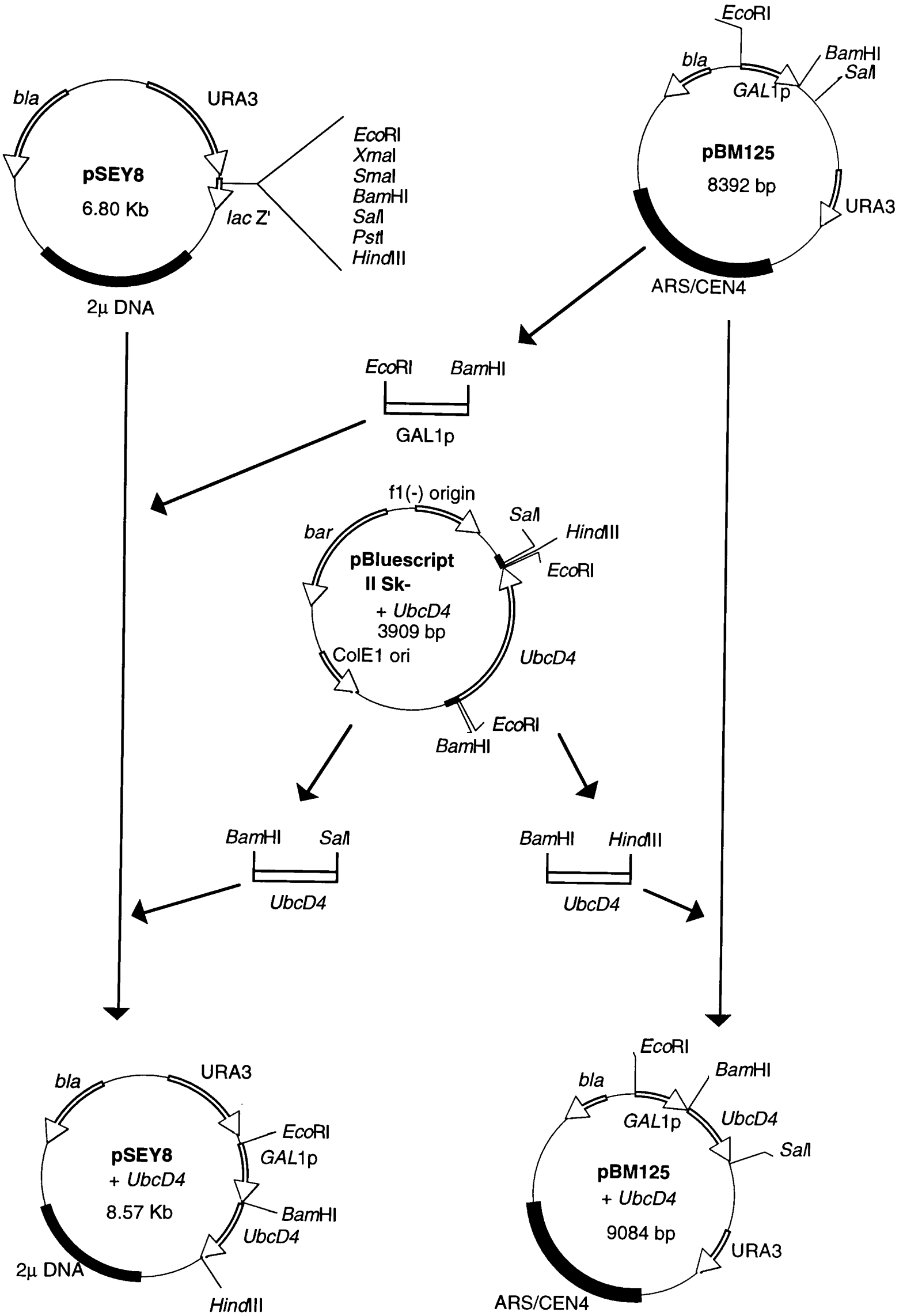


Fig. 3.19 Construction of plasmids for overexpression of UBCD4 in yeast strain YW023 *ubc4ubc5*.

3.3 DISCUSSION:

A new *Drosophila* E2 gene named *UbcD4* was cloned and sequenced. It is surprising that only one band was seen using N54 and N56 as primers, but perhaps the primers bound well to *UbcD4*, and less strongly to other E2 enzyme sequences. The other set of degenerate primers produced multiple bands, only one of which was part of an E2 enzyme. These primers were designed to anneal to regions from all E2s, but only amplify fragments from a subset of E2s. If different regions of E2 enzymes were used to design primers to anneal to all E2s, perhaps a different subset of E2 enzymes would be cloned.

UBCD4 is the *Drosophila* homologue of bovine $E2_{25K}$. When multiple alignments of the UBC domains of all E2 enzymes in the SwissProt database were made, the resulting dendrogram showed that both proteins are related to UBC1. *UbcD4* may not be able to substitute for UBC1 in rescuing the *ubc4ubc5* yeast strain YW023, but this was not proved, as no experiments were performed to show UBCD4 protein was expressed. Other *Drosophila* E2s that are able to rescue yeast mutants are more closely related to each other than UBCD4 is to UBC1. *UbcD1* can fully restore growth of *ubc4ubc5* mutants at 37°C (Treier *et al.*, 1992), *UbcD2* only partially complements the growth defects, and *UbcD3/ben* cannot restore growth (Matuschewski *et al.*, 1996). It seems likely *UbcD4* would at best be able to partially restore growth of YW023 yeast cells at 37°C, as it is less related to *UBC4* and *UBC5* than *UbcD2* (figure 3.18).

The dendrogram of figure 3.18 could be used to determine sub-groups of E2 enzymes. *UbcD1* lies within the yeast UBC4/UBC5 cluster, as expected, and *Dhr6* is found with other UBC2 homologues. *UbcD2* appears to be a distant relative to the UBC4/UBC5 subgroup, and *ben* may be related to UBC1-like E2s.

Genomic Southerns show *UbcD4* is a unique gene, but bands not from *UbcD4* are seen to hybridise weakly to the probe. It is possible that they correspond to a related gene, as UBC1, 4 and 5 homologues have been found to be encoded by multigene families in other organisms, such as *Arabidopsis* (Girod et al., 1993).

Knowing the position of the *UbcD4* gene within the genome could be useful when trying to create mutants of this gene. Many stocks are available with P elements residing in different positions within the *Drosophila* genome, and a P element lying near *UbcD4* could be mobilised to create insertional mutations. Also deletions covering this region could reveal the mutant phenotype of the gene in conjunction with another mutant allele for *UbcD4*, such as a P element insertion.

CHAPTER 4

RNA STUDIES ON *DROSOPHILA* E2 ENZYMES

4.1 INTRODUCTION:

Ubiquitin-dependent proteolysis may play an important role during early *Drosophila* development, as nurse cells and follicle cells undergo programmed cell death in oogenesis, and in the embryo certain regulatory proteins need to be transiently expressed and then rapidly degraded, such as those establishing the embryonic axes. Ubiquitin is involved in degrading cyclins and controlling the cell cycle (see Chapter 1). The first 10 mitotic divisions in the *Drosophila* embryo require different control to later divisions, as they occur in a syncitium and are very rapid. Programmed cell death also occurs during metamorphosis in the pupa, when larval tissues are destroyed, and the adult forms from imaginal discs.

There are many E2 enzymes present in an organism, as one means of providing substrate specificity for the ubiquitin system (see chapter 1). It is likely some ubiquitin conjugating enzymes have specific roles in development. In order to investigate temporal and spatial control of expression of *Drosophila* E2s during development, RNA studies were performed.

4.2 RESULTS:

4.2.1 NORTHERN ANALYSIS:

The first step in investigating developmental roles for cloned *Drosophila* E2 enzymes was to perform developmental Northerns. These were used to determine the size and abundance of transcripts, and to show how levels of mRNA vary during development. If a large amount of transcript from an E2 is present at a certain stage of development, this could imply the enzyme has a specific developmental function at this stage.

4.2.1.1 *UbcD1*, *UbcD2*, *ben*, *UbcD4* and *Dhr6* are all expressed in adult *Drosophila*:

In order to confirm the size and number of transcripts of *UbcD1*, *UbcD2*, *ben* and *Dhr6* (published in Treier *et al*., 1992, Matuschewski *et al*.., 1996, Muralidhar and Thomas, 1993, and Koken *et al*., 1991a. respectively), and to see the size and number of *UbcD4* mRNAs, Northerns were performed on total adult OregonR (OrR) RNA (10μg per sample). Ubiquitin conjugating enzyme cDNAs were ^{32}P-labelled and used as probes.

All E2s were expressed in adult RNA. *UbcD1*, *UbcD2* and *UbcD4* were seen

to have transcripts of 1.5, 1.6 and 1.8kb respectively (figure 4.1). *Dhr6* and *ben* each have two transcripts present in adult RNA. As published, *ben* mRNAs are 1.2 and 2.0kb, and those of *Dhr6* are 1.3 and 2.1kb (figure 4.1).

4.2.1.2 Developmental Northerns:

Different developmental stages of wild-type OrR *Drosophila melanogaster* were collected as described in 2.3.3.1, and total RNA preparations were made. Approximately 10μg total RNA were loaded per lane on each gel, and Northern blots were performed, again using ^{32}P-labelled E2 cDNAs as probes. After exposure to film, each radioactive filter was exposed to a phosphoimager cassette, then stripped and reprobed with α-tubulin. This was used as a loading control, as it has been shown to be expressed at a constant level throughout development (Kalfayan and Wensink, 1982), although expression levels in different adult tissues are unknown, so this was not a good standard for comparing ovarian E2 mRNA levels to those of different developmental stages. Again filters were exposed first to film, then in a phosphoimager cassette. This enabled quantitative measurements to be made on the way levels of E2 enzyme mRNAs vary during development.

All E2 transcripts seen in adult RNA were detected throughout development (figures 4.2-4.6). For all E2 enzymes, transcript levels in ovaries appeared low. This could be due to many α-tubulin transcripts being present in this tissue. *UbcD1* and *UbcD4* seemed to show a higher level of expression than the other E2s in general, though this could only be confirmed by using probes with the same specific activity and performing Northern blots under identical experimental conditions. *UbcD4* hybridises to more than one band in the developmental Northern (figure 4.5). It is possible the extra bands correspond to transcripts from other similar E2 enzymes, as UBC1, 4 and 5 homologues have been found to be encoded by multigene families in other organisms (see 1.7.1.1). The smaller 1.2kb *ben* transcript was usually present at higher levels than the larger 2.0kb transcript, while both *Dhr6* mRNAs were present at approximately equal levels.

Quantitative analysis revealed levels of transcripts varied up to 26 fold between stages for certain E2s (table 4.1 and figure 4.7). In order to determine the significance of the observed differences, these experiments would have to be repeated and statistical analysis performed on the sets of data. *UbcD4* transcript levels were more or less constant, allowing for some degree of experimental error in these values. The highest levels for each of the other E2s tested were found in larvae, whilst pupal levels were low for all except *UbcD1*. *UbcD1* showed high expression

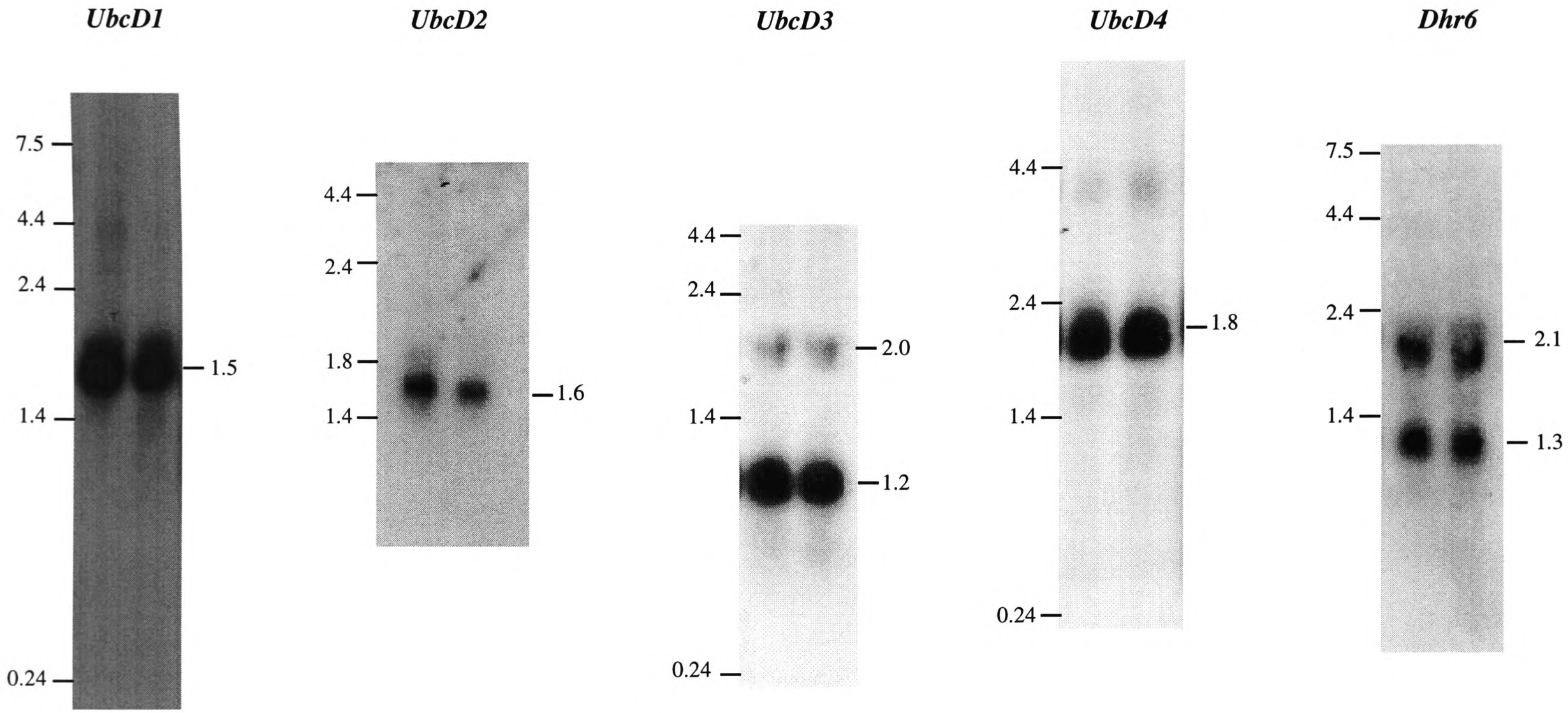
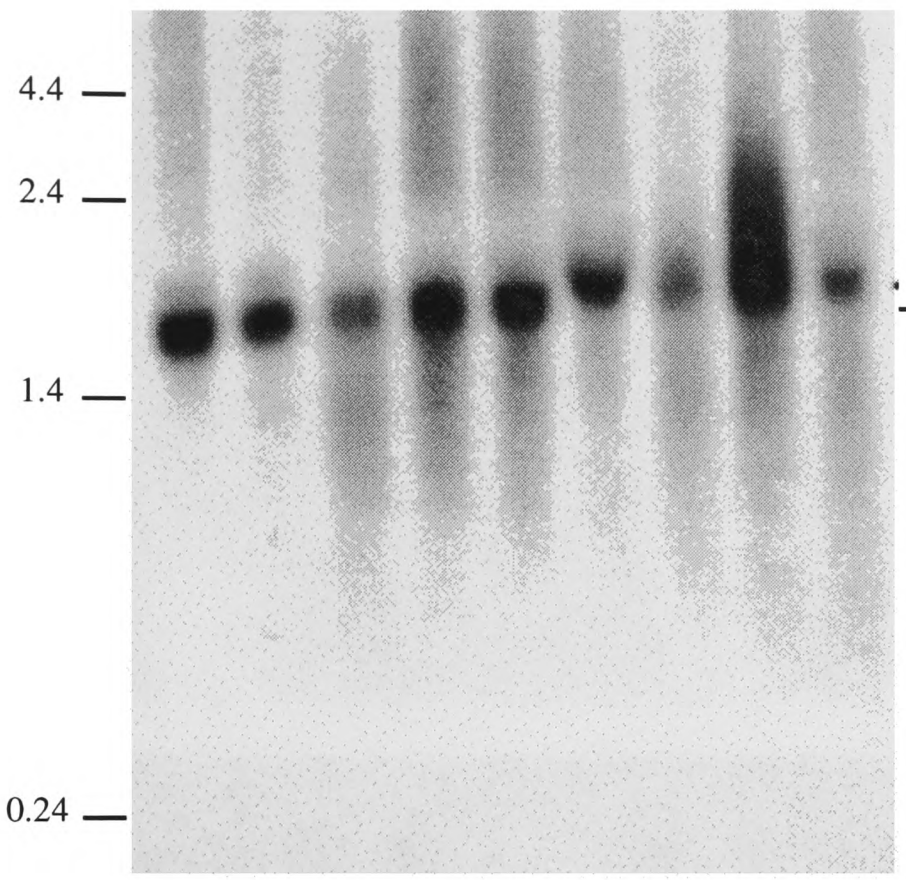


Fig. 4.1 Northerns of total adult RNA to show the number and size of transcripts for *Drosophila* E2 enzymes.
Sizes are in kb. RNA ladder used for size markers (Boehringer Mannheim).
Approximately 10µg total RNA were loaded per lane.



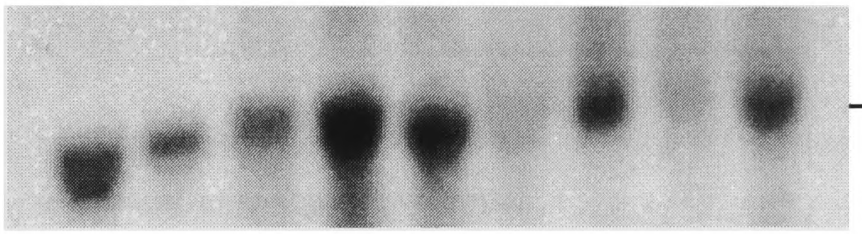


Fig. 4.2 *UbcD1* developmental Northern of total RNA from different stages. α-tubulin was used as a loading control.

Sizes are shown in kb. Boehringer Mannheim RNA ladder was used as size markers. Approximately 10μg total RNA were loaded per lane.

(O = ovaries; E0-2 = embryos 0-2h; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6h; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males).

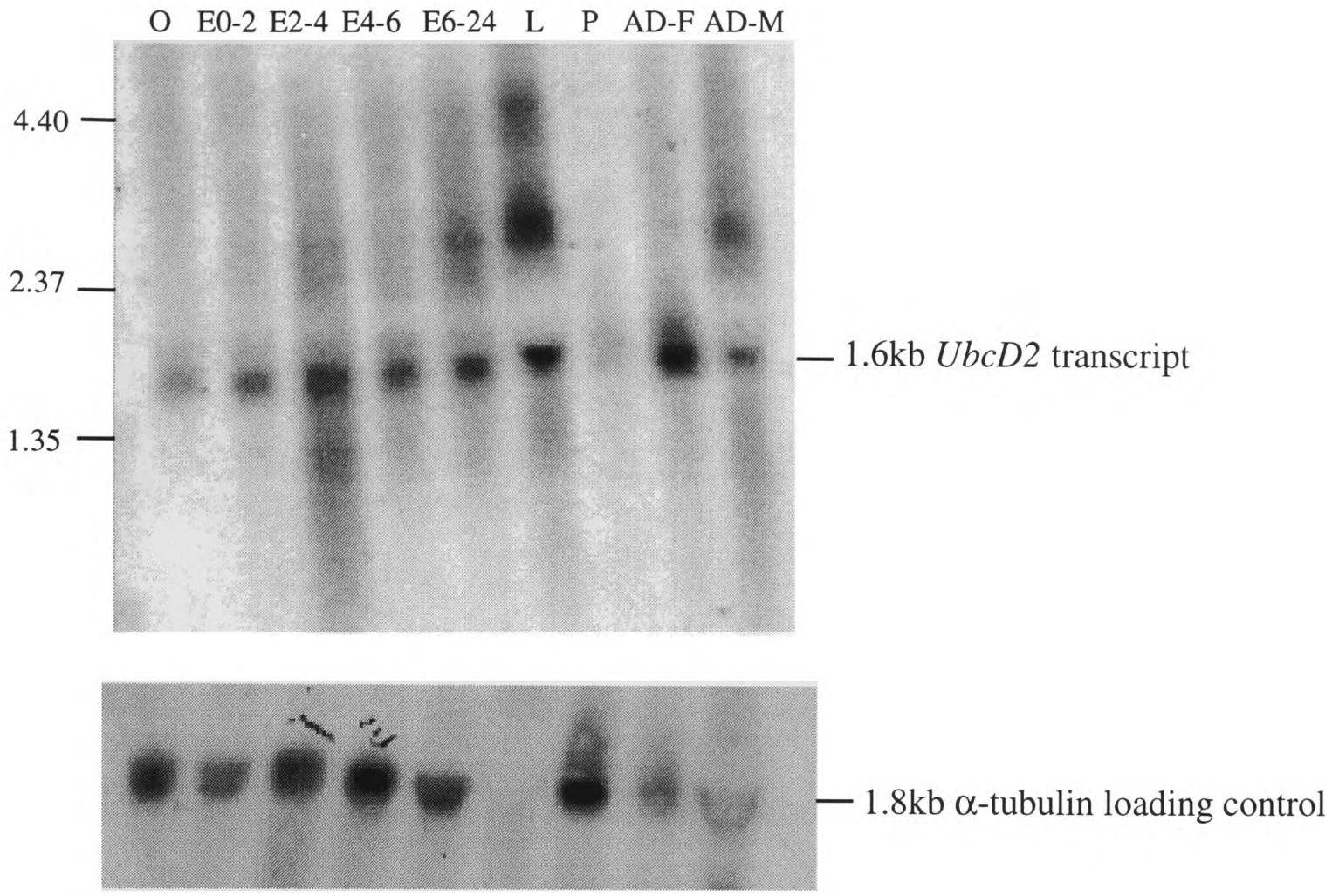


Fig. 4.3 *UbcD2* developmental Northern of total RNA from different stages. α-tubulin was used as a loading control.

Sizes are shown in kb. Boehringer Mannheim RNA ladder was used as size markers. Approximately 10μg total RNA were loaded per lane.

(O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males).

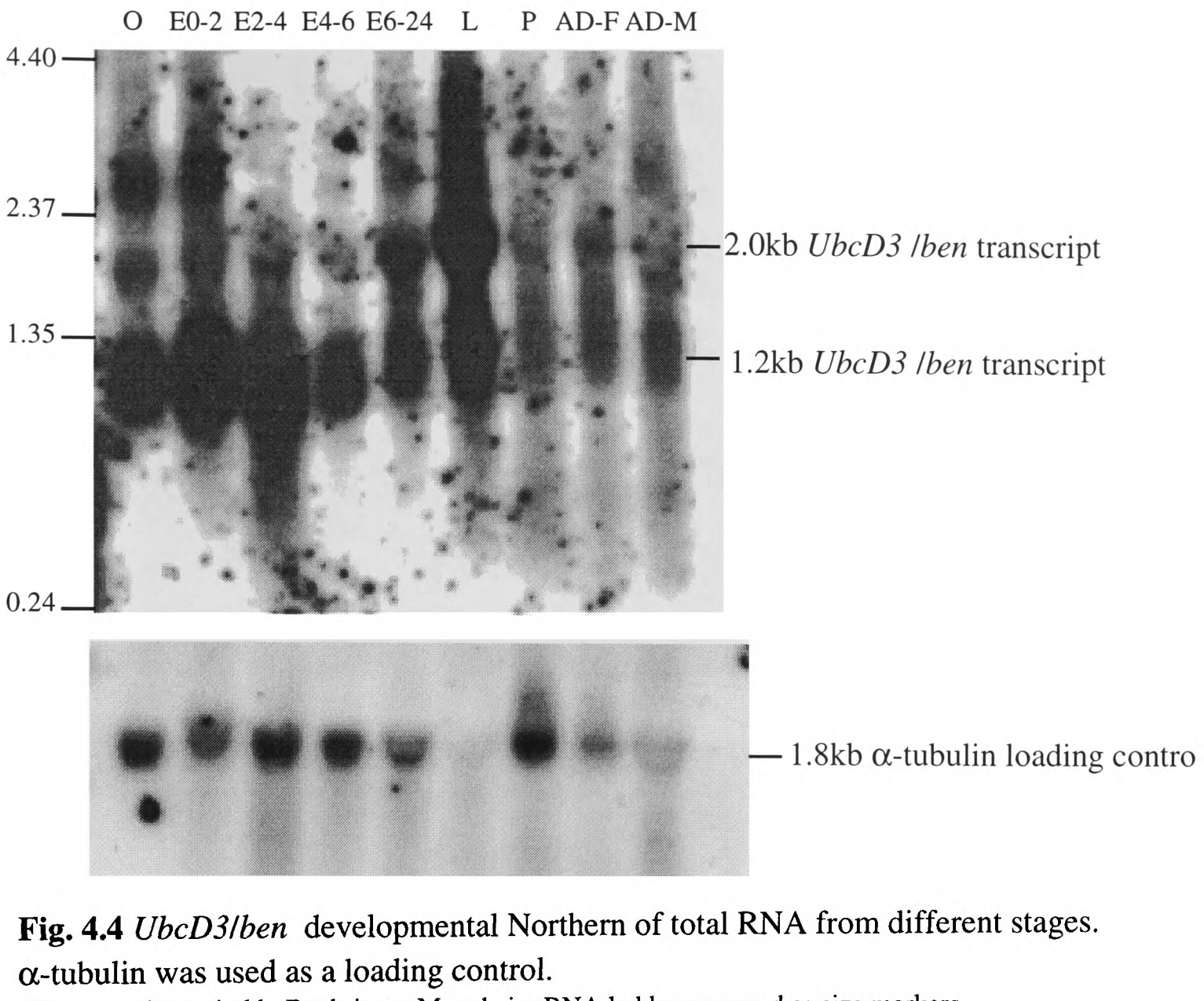


Fig. 4.4 *UbcD3/ben* developmental Northern of total RNA from different stages. α-tubulin was used as a loading control.

Sizes are shown in kb. Boehringer Mannheim RNA ladder was used as size markers. Approximately 10μg total RNA were loaded per lane.

(O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males).

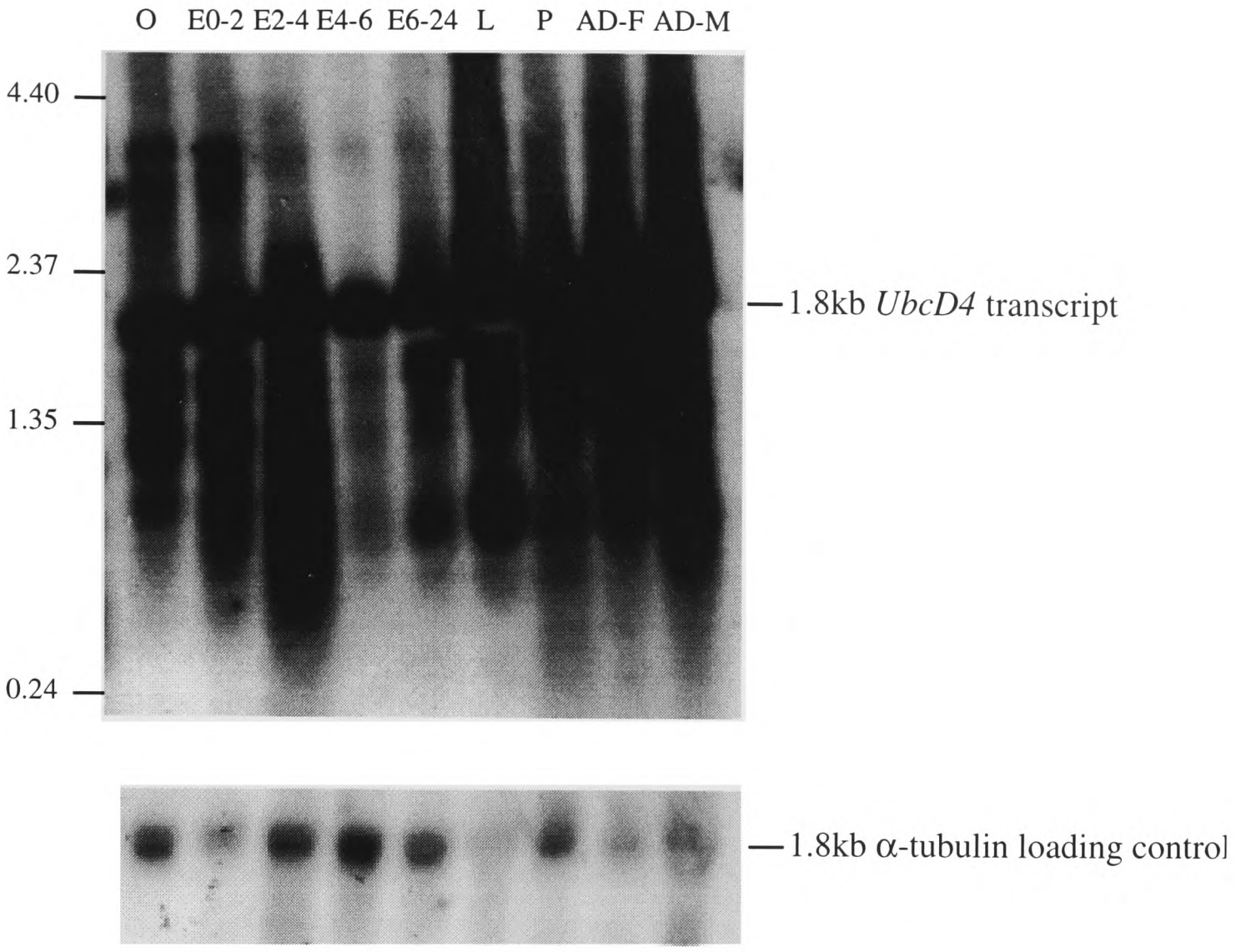


Fig. 4.5 *UbcD4* developmental Northern of total RNA from different stages. α-tubulin was used as a loading control.

Approximately 10μg total RNA were loaded per lane.

Sizes are shown in kb. Boehringer Mannheim RNA ladder was used as size markers.

(O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males).

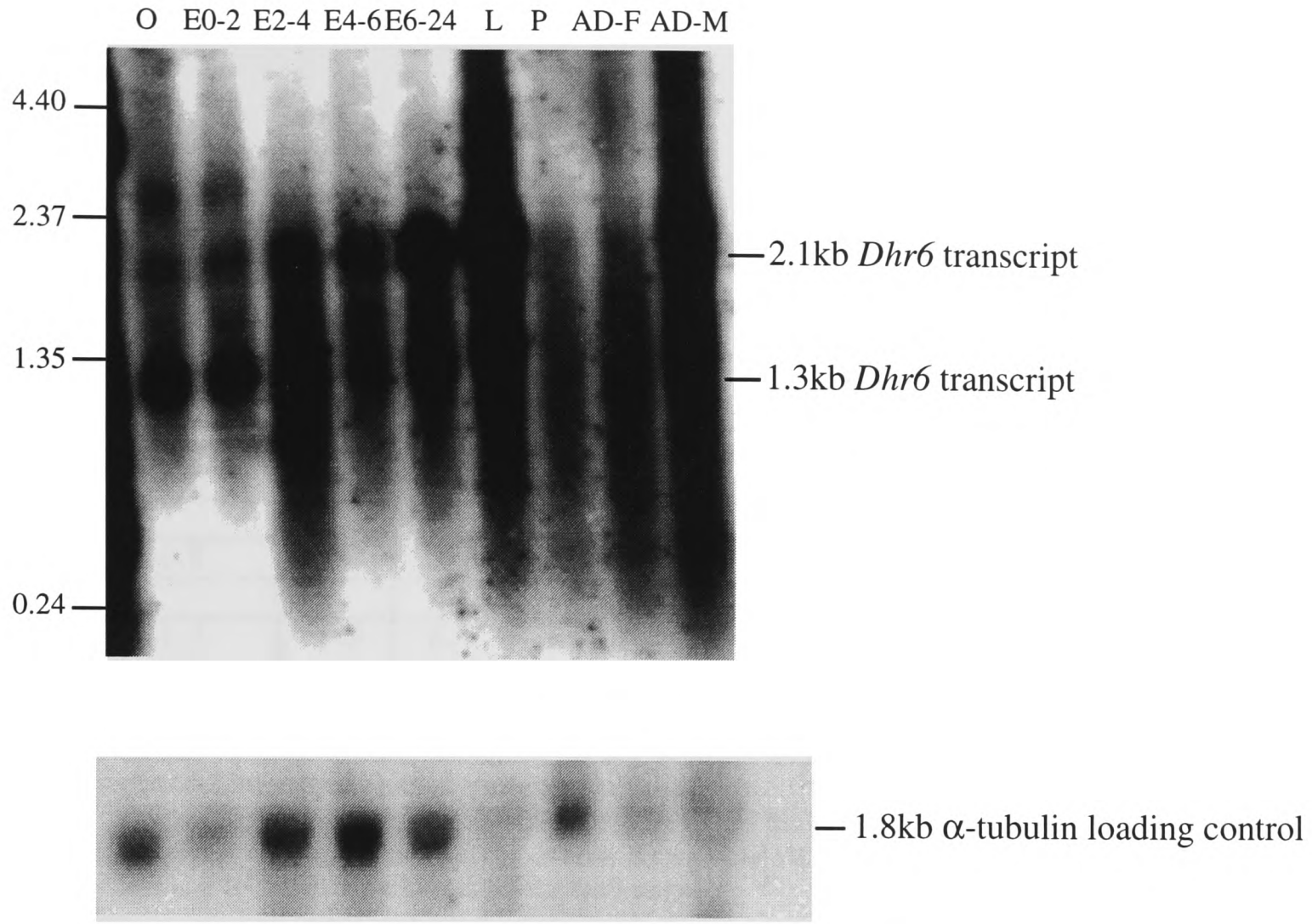


Fig. 4.6 *Dhr6* developmental Northern of total RNA from different stages. α-tubulin was used as a loading control.
Approximately 10μg total RNA were loaded per lane.
Sizes are shown in kb. Boehringer Mannheim RNA ladder was used as size markers.
(O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males).

Gene	mRNA (kb)	O	E0-2	E2-4	E4-6	E6-24	L	P	AD-F	AD-M
UbcD1	1.5	1.0	3.5	0.8	0.8	1.4	11.5	1.1	7.5	2.9
UbcD2	1.6	1.0	2.8	1.3	0.7	0.7	13.1	0.5	2.7	1.9
ben	1.2	1.0	3.8	1.6	0.6	0.6	13.4	0.1	0.8	0.7
	2.0	1.0	0.9	0.5	0.4	1.6	26.7	0.5	1.4	1.7
UbcD4	1.8	1.0	2.1	1.1	2.7	1.3	1.0	1.0	3.4	1.6
Dhr6	1.3	1.0	8.3	0.9	0.3	0.8	6.7	0.3	1.2	1.8
	2.1	1.0	10.1	1.4	1.0	6.6	11.7	0.4	1.4	2.9

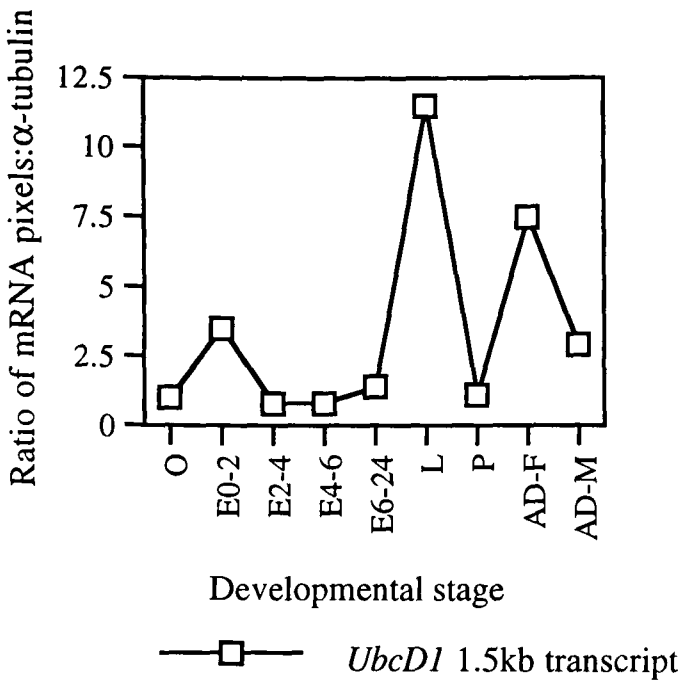
Table 4.1 Results of quantitative analysis on the amount of E2 mRNA present at different stages of *Drosophila* development.

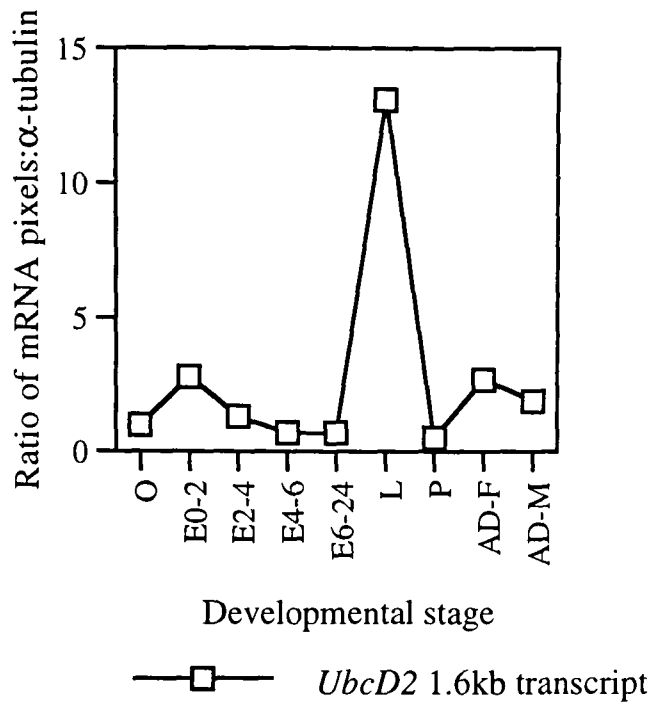
Values were calculated using the Volume Integration programme on Molecular Dynamics ImageQuant software. This calculates the number of pixels in an area. Background values were deducted, and ratios between α-tubulin and E2 bands were calculated for each stage.

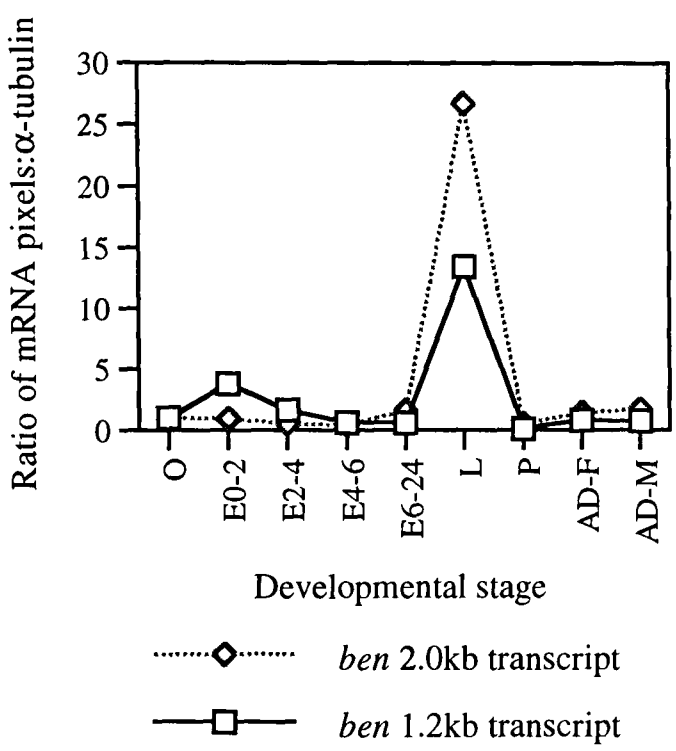
In order to directly compare ratios between different stages within a particular developmental Northern, ovary E2 band:α-tubulin ratios were taken to be 1.0. However, α-tubulin is not a good loading control for comparing ovary expression to that of different developmental stages, as the level of expression between different tissues is not known. It appears the level of expression of E2 mRNA is low in ovaries, but this is probably not the case.

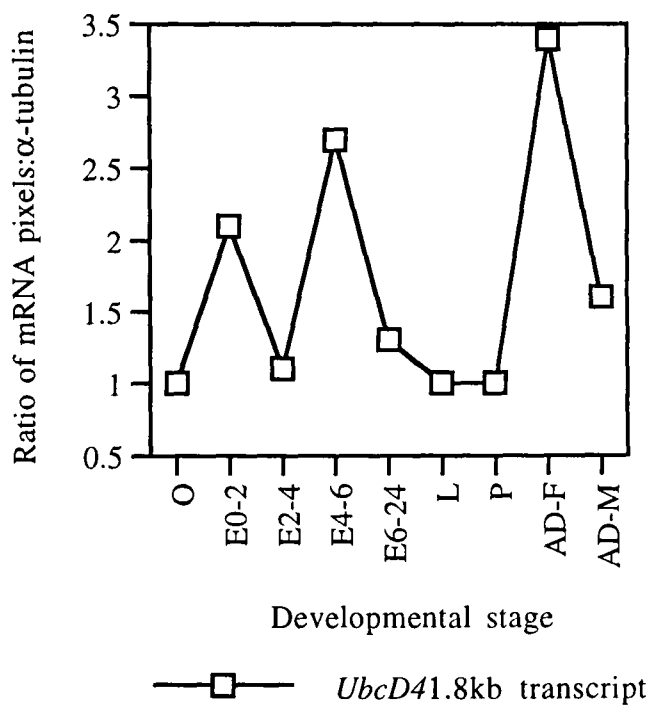
Values cannot be compared between different E2 enzymes.

O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males.









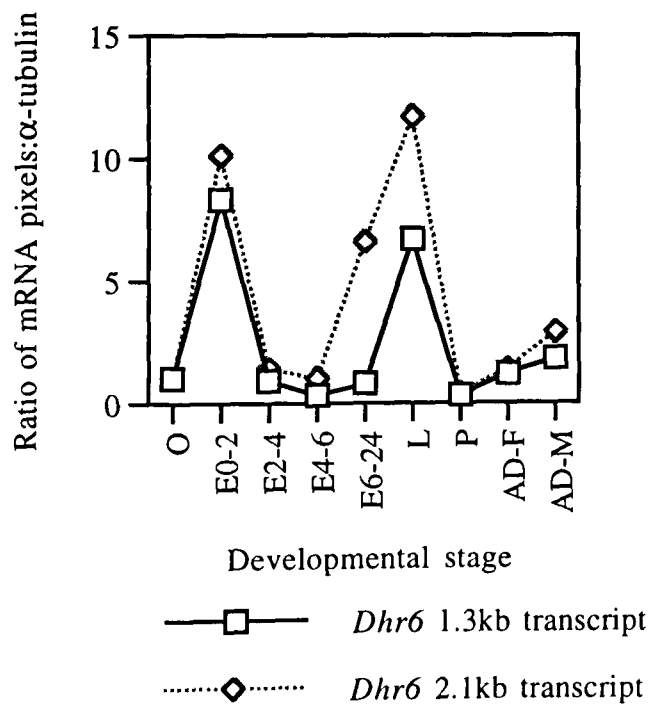


Fig.4.7 Graphs to show variation in levels of E2 enzyme transcripts across development.
Data from table 4.1.
The amount of E2 mRNA in ovaries may appear low due to large amounts of α-tubulin transcripts being present.
O = ovaries; E0-2 = embryos 0-2hr; E2-4 = embryos 2-4hr; E4-6 = embryos 4-6hr; E6-24 = embryos 6-24hr; L = larvae; P = pupae; AD-F = adult females; AD-M = adult males.

in adult females, and elevated expression in 0-2hr embryos. These were the two other stages where increased levels of *UbcD2* mRNA were detected. Both *ben* transcripts showed similar patterns of expression, except in 0-2hr embryos where the 1.2kb mRNA showed an increase in abundance. *Dhr6* mRNAs increase in number for both transcripts at the 0-2hr embryo stage as well as in larval stages. Again both transcripts show similar variations except at the 4-6hr embryo stage where the 2.1kb transcript increases in number greater, with respect to other stages, than the 1.3kb mRNA.

4.2.1.3 Discussion:

All E2 transcripts investigated are present in adult RNA. The size and number of *UbcD1*, *ben* and *Dhr6* transcripts were confirmed. *UbcD2* was seen to have one transcript of approximately 1.6kb, although Matushchewski *et al.*. (1996) report an approximately 1.5kb transcript. The exact size is probably between these two values. *UbcD4* has a 1.8kb transcript. This is different from the expected size of 950bp from the cDNA clone, perhaps due to the cDNA clone not being complete. There is no sequence matching the polyadenylation signal consensus in the genomic sequence at the site corresponding to the 3' end of the cDNA, while sequences at the 5' end matching the TATA box, CAAT box and transcription start site are present (see chapter 3). This suggests the 3' end of the cDNA clone is not complete.

UbcD1 and *UbcD4* mRNAs appear to be present at higher levels across development than other E2s tested, although this has not been confirmed. They may be required at constantly high levels due to their putative roles as enzymes for bulk degradation of abnormal and short-lived proteins (Treier *et al.*, 1992; chapter 3). A fairly constant level of *UbcD4* mRNA is seen at all stages, again possibly due to the enzyme functioning in a wide variety of ubiquitin conjugation reactions. *UbcD4* cDNA hybridised to more than one band in developmental Northerns. The higher band of approximately 4kb also hybridised weakly in adult Northerns (figure 4.1), and may correspond to a transcript from a similar E2 enzyme, such as the putative E2 which hybridises weakly to *UbcD4* cDNA in genomic Southerns (figure 3.13). Smaller bands may be due to degradation of RNA.

The highest levels of mRNA for E2s, other than *UbcD4*, were found at the larval stage of development. This implies an increase in activity of the ubiquitin pathway at this stage. In larvae, most larval tissue cells grow bigger without dividing, and the majority of cells determined to give adult tissue are segregated and

organised into imaginal discs. The cell cycle is therefore altered, and transcription patterns within cells will be altered, but it is not obvious why an increase in protein degradation via the ubiquitin pathway would be necessary. Development of the giant fibre and retinal innervation of optic lobes occurs at late third larval and early pupal stages, so *ben* must be active, and this could be why high levels of *ben* mRNA are observed in larvae.

A lot of protein degradation takes place in the pupa, when larval tissues are destroyed and the adult forms from imaginal discs. Levels of mRNA from E2 enzymes were seen to fall at the pupal stage, implying less activity of the ubiquitin system. This may be a function of the difficulty in obtaining good pupal RNA. Often pupal RNA was found to be degraded, possibly due to the amount of degradation occuring naturally in the pupa. However, were this to be the case, α-tubulin RNA would be likewise affected.

High levels of mRNA for *UbcD1*, *UbcD2*, *ben* 1.2kb transcript, and *Dhr6* transcripts were seen in 0-2hr embryos. This probably reflects maternal mRNA, as embryonic transcription would not have been expected to have taken place. The embryo may inherit mRNA for ubiquitin conjugating enzymes, in order to be able to rapidly degrade proteins, such as bicoid, which are necessary only transiently to establish embryonic axes. *Dhr6* is the homologue of yeast RAD6. This protein has been seen to have a cell cycle component (Silver *et al.*, 1992) so the *Dhr6* transcripts may have a role in the early rapid nuclear divisions of embryogenesis.

Where adult females show high values for amounts of E2 mRNA, and where they are higher than adult males, this may reflect the amount of E2 transcripts in ovarian tissue. Follicle cells and nurse cells undergo programmed cell death (Mahowald and Kambysellis, 1980) so ubiquitin conjugating activity may be important here.

Matuschewski *et al.* (1996) also found *UbcD1* and *ben* transcripts throughout development (results not shown). In contrast to my results, they found *UbcD2* appeared to be expressed exclusively at post-larval stages (results not shown). Both my results and those of Muralidhar and Thomas (1993) showed *ben* to have two transcripts of 1.2kb and 2.0kb. Euk Oh *et al.* (1994) found a 1.9kb transcript expressing throughout development with heightened expression at the pupal stage. However, they did not have a control to show equal loading of lanes on the gel.

Values for levels of mRNA do not necessarily reflect levels of enzyme activity, but mRNA levels from several E2 enzymes do vary across development. In

particular, stages at which the ubiquitin system may be playing important roles are in larvae, 0-2hr embryos, and adult females, probably in the ovaries. For the variations in transcript levels to be determined as significant, these experiments must be repeated.

4.2.2 *IN SITU* HYBRIDISATION TO EMBRYO RNA:

E2 enzymes could have distinct developmental roles due to differential expression in different tissues. High levels of *ben* mRNA are restricted to the CNS, where it is known to be required, during development (Muralidhar and Thomas, 1993). RNA *in situ* hybridisation to wholemount embryos was used to show where other E2 mRNAs were located throughout embryonic development.

4.2.2.1 *UbcD1*, *UbcD2*, *ben* and *Dhr6* mRNAs become restricted to the central nervous system (CNS) during embryonic development:

Each E2 cDNA was labelled with DIG (see 2.3.4.1), and used as a probe to detect mRNA *in situ*, in fixed wholemount OrR embryos collected over 24hr. DIG is detected by anti-DIG antibodies which are conjugated to alkaline phosphatase. Sites of cDNA hybridisation were detected as a purple colour after developing with X-phosphate and NBT. To confirm that signals were due to hybridisation of the probes, control *in situ* hybridisations were performed. When no probe was used, no staining was seen (figure 4.8). The positive control used was *fushi tarazu* (DNA provided by Dr L. Brown), and this gave the expected 7 stripes (Carroll and Scott, 1985) of hybridisation (figure 4.9).

UbcD1 mRNA was present throughout the embryo at pre-blastoderm and blastoderm stages (figure 4.10). This includes the 0-2hr embryo stage when *UbcD1* mRNA levels are seen to be high (figure 4.7). From gastrulation through to stomodeal invagination, mRNA is restricted to the presumptive mesoderm (figure 4.10) and areas which will develop into the nervous system (3-6hr embryo). High levels of mRNA are restricted to the developing CNS at later stages (figure 4.10).

This is the pattern of expression seen for *ben* (Muralidhar and Thomas, 1993), and the same pattern was observed when *UbcD2* (figure 4.11), *ben* (figure 4.12) and *Dhr6* (figure 4.13) were used as probes.

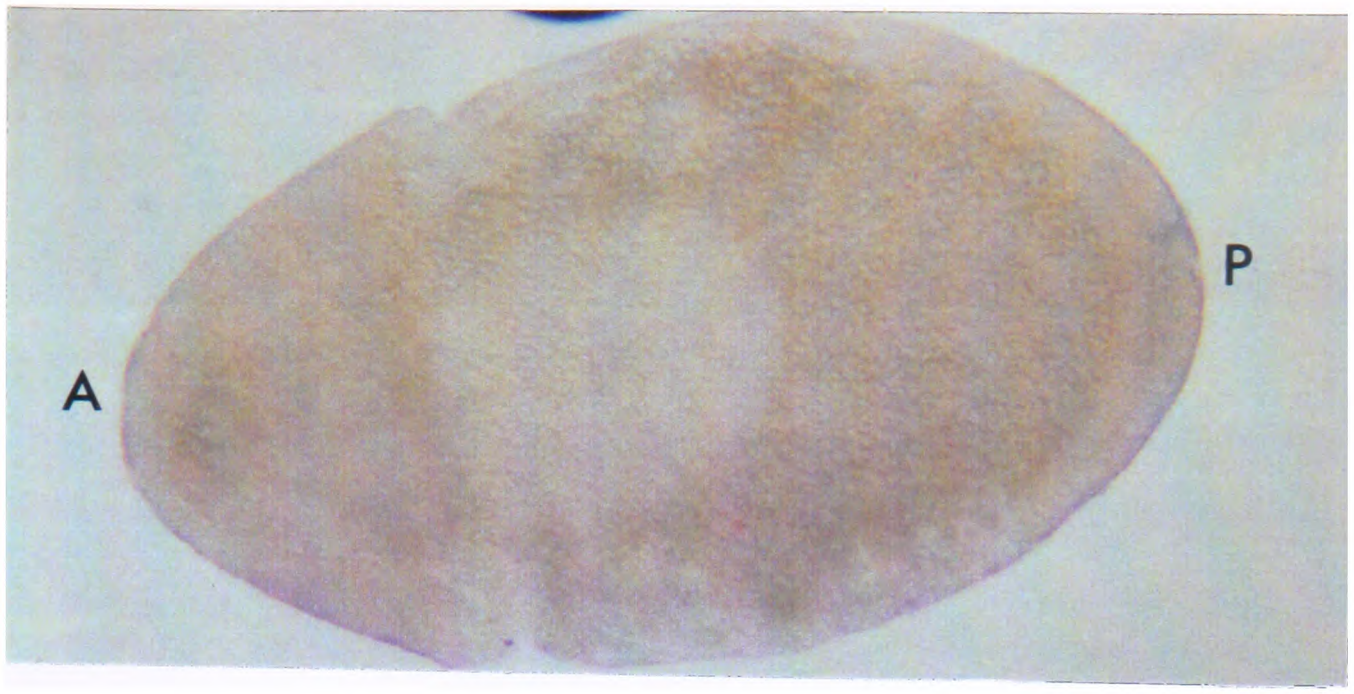


Fig. 4.8 OrR embryo taken through the RNA *in situ* process with no probe.
Stage 7: midgut invagination.

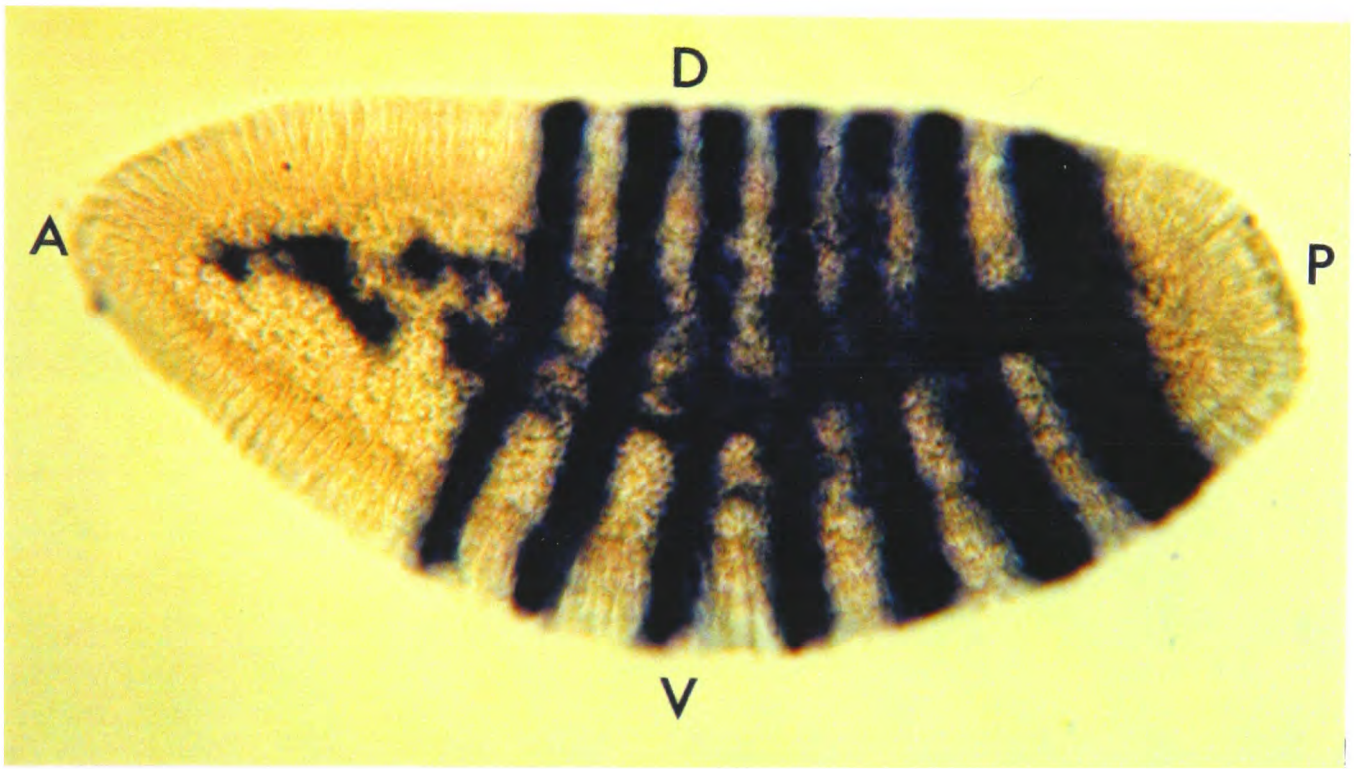
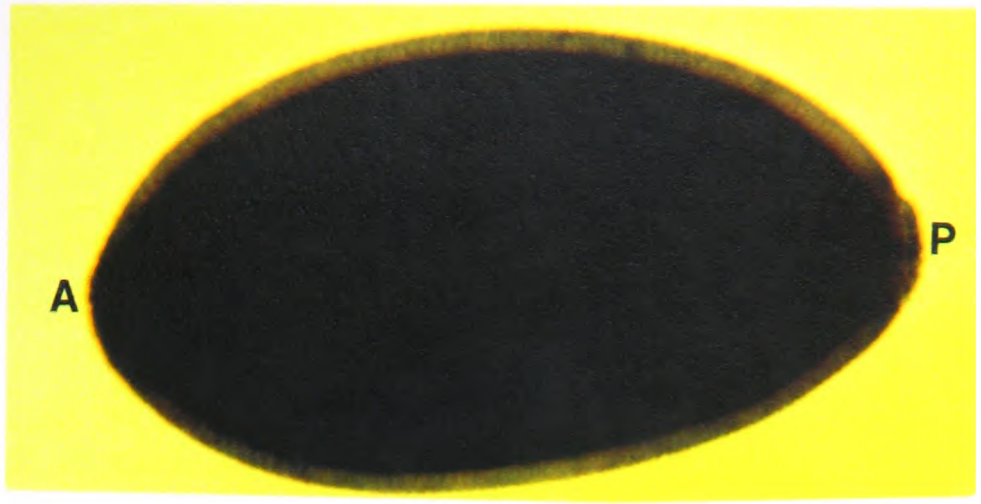


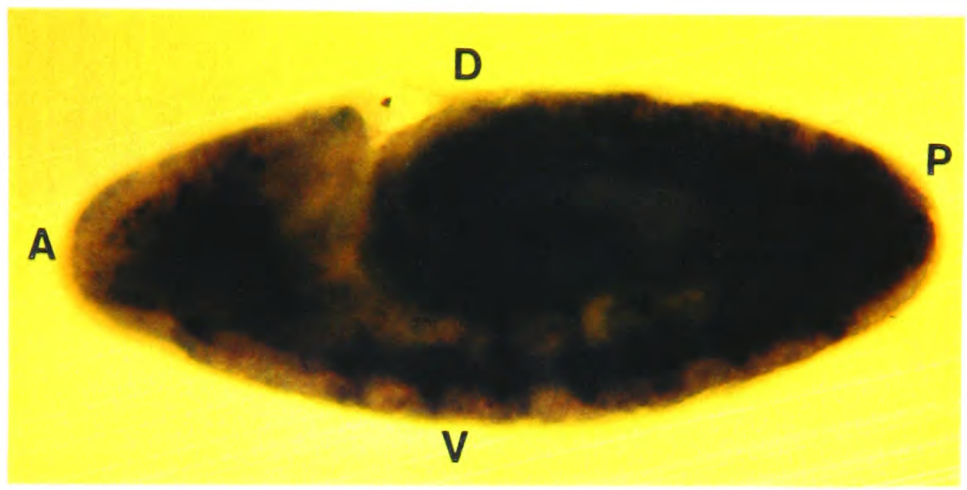
Fig. 4.9 RNA expression pattern of *ftz*.
Stage 5: cell formation.

A= anterior; P= posterior; D= dorsal; V= ventral

Stage 5.
Cell
formation.

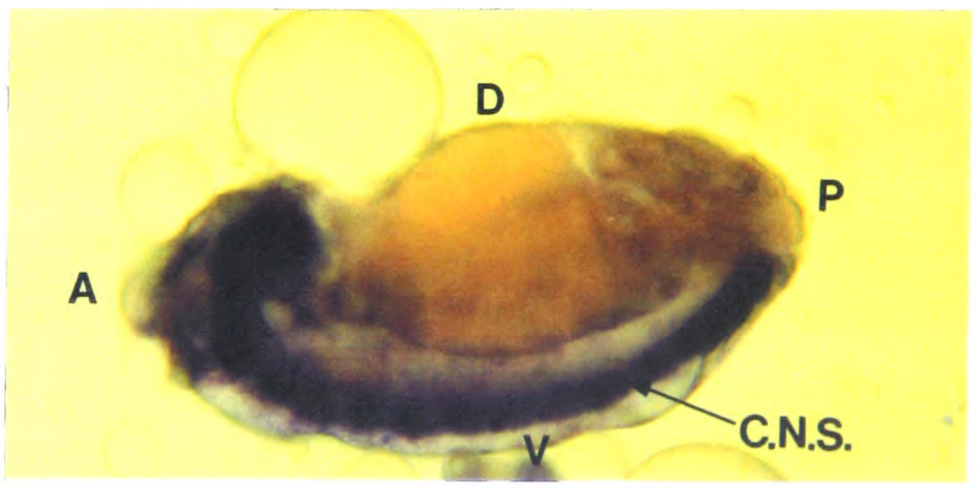


Stage 9.
Stomodeal plate
formation.



Stage 15.
Dorsal closure
complete.

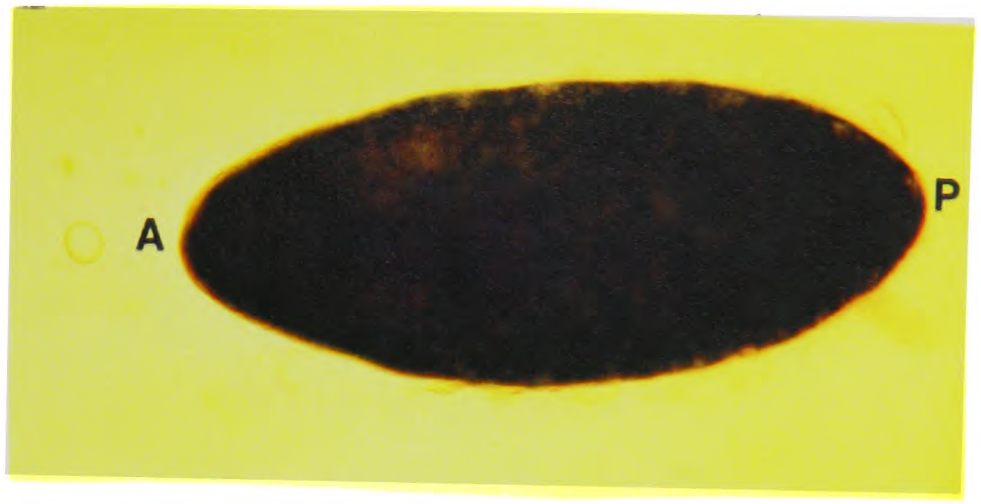
Stage 15.
Dorsal closure
complete.



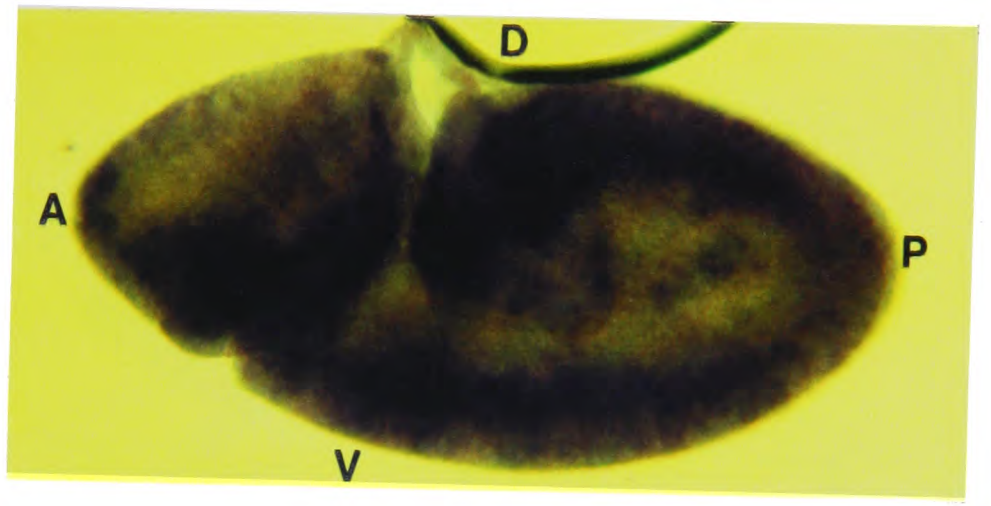
A= anterior; P= posterior; D= dorsal; V= ventral.

Fig. 4.10 *UbcD1* RNA *in situ* hybridisation to wholemount OrR embryos.
Stages from Wieschaus and Nusslein-Volhard, 1986.

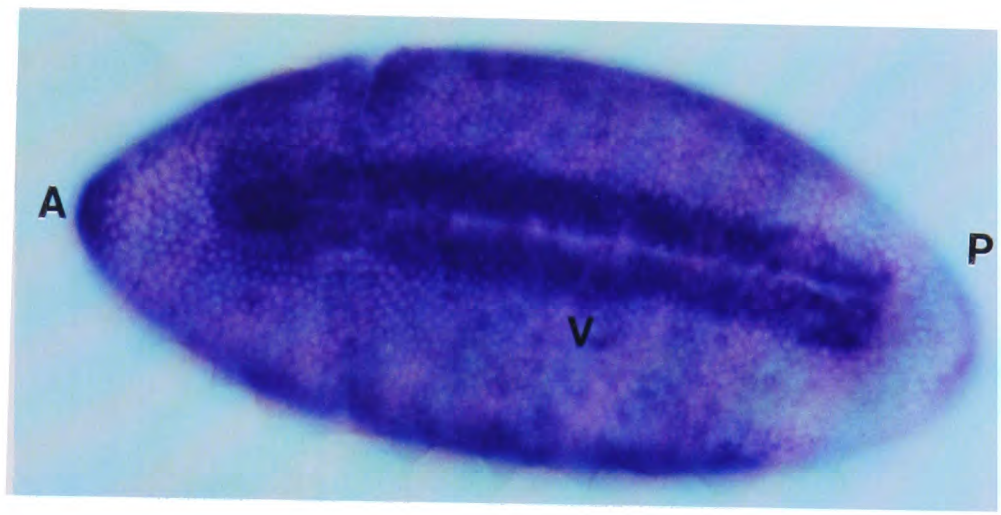
Stage 1.
Freshly laid egg.



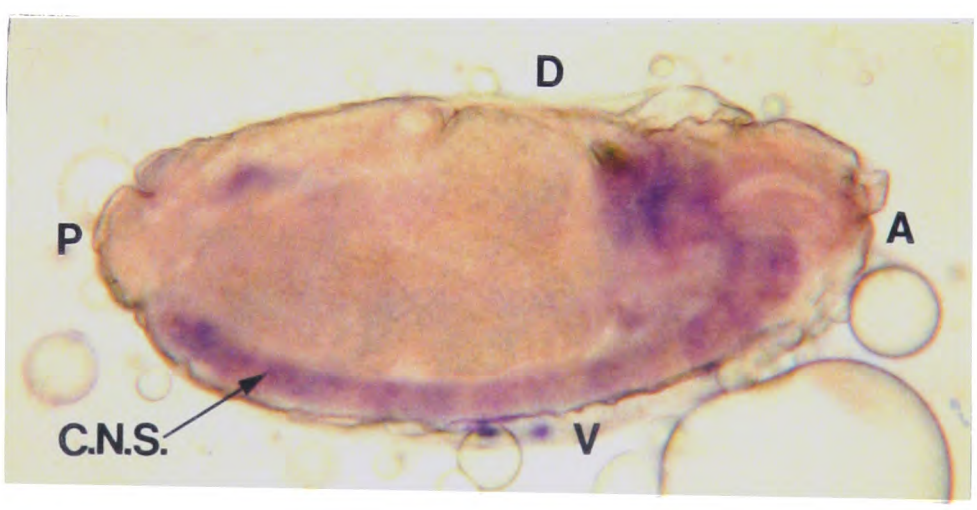
Stage 8.
Germ band extension.



Stage 12.
Shortening of germ band.



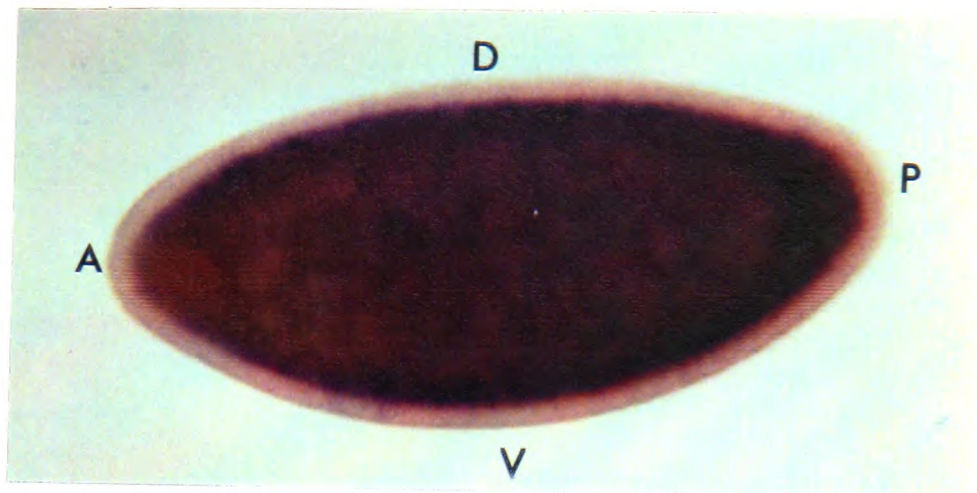
Stage 15.
Dorsal closure complete.



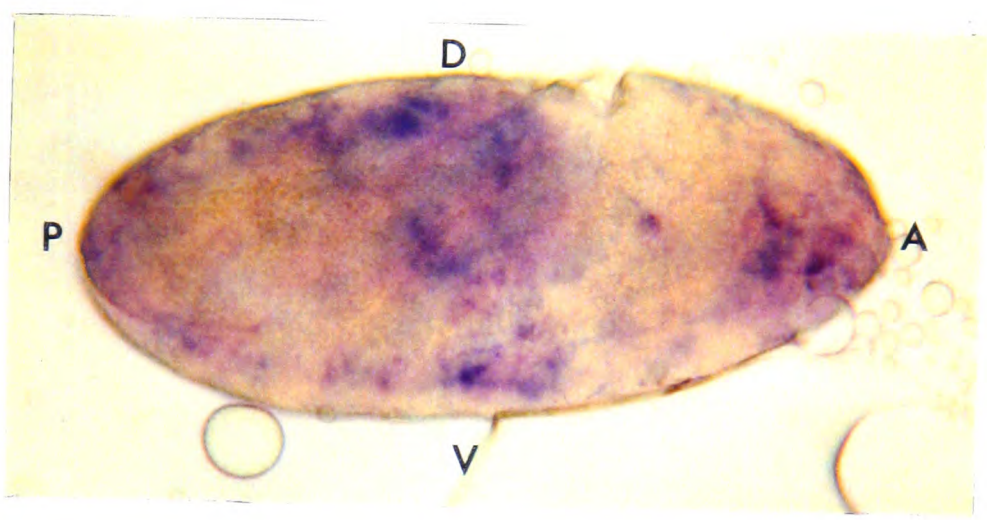
A= anterior; P= posterior; D= dorsal; V= ventral.

Fig. 4.11 *UbcD2* RNA *in situ* hybridisation to wholemount OrR embryos.
Stages from Wieschaus and Nusslein-Volhard, 1986.

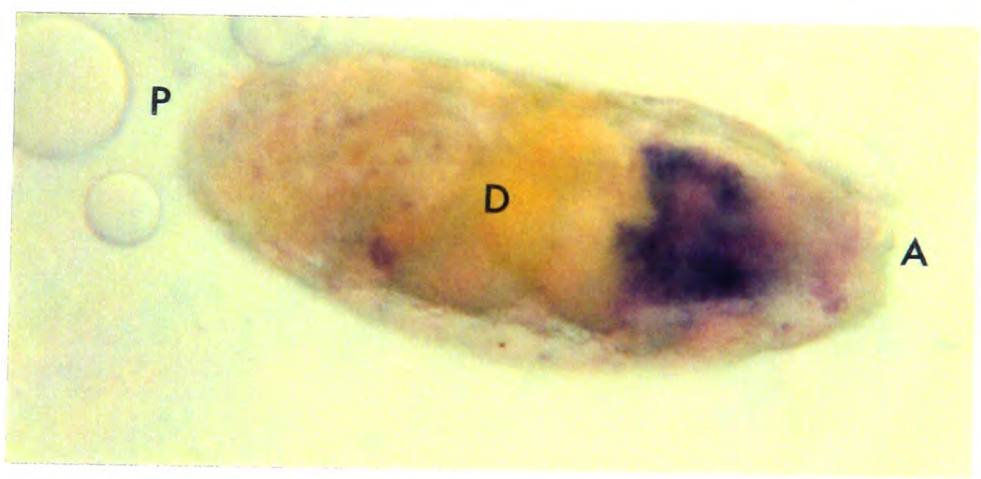
Stage 5.
Cell
formation.



Stage 8.
Germ band
extension.



Stage 15.
Dorsal closure
complete.

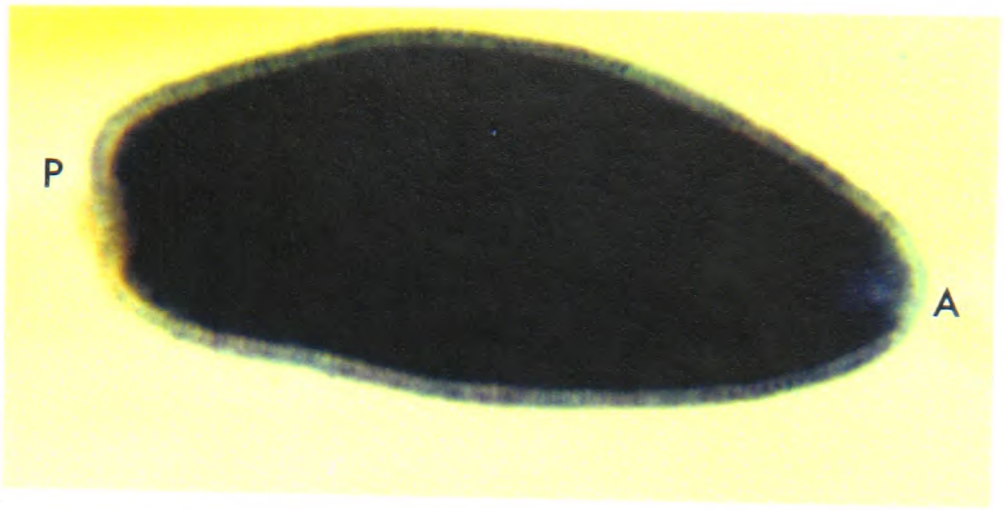


Stage 16.
Condensation
of C.N.S.

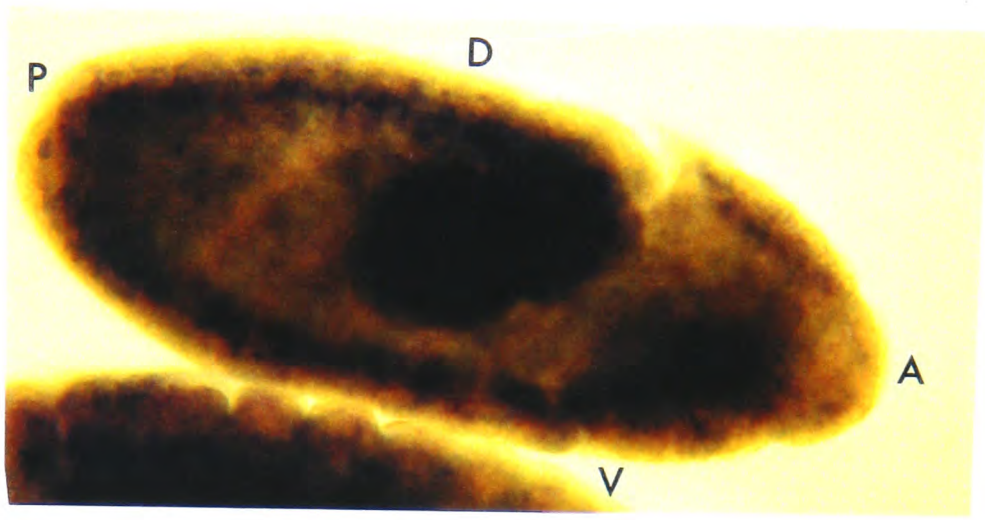
A= anterior; P= posterior; D= dorsal; V= ventral.

Fig. 4.12 *ben* RNA *in situ* hybridisation to wholemount OrR embryos.
Stages from Wieschaus and Nusslein-Volhard, 1986.

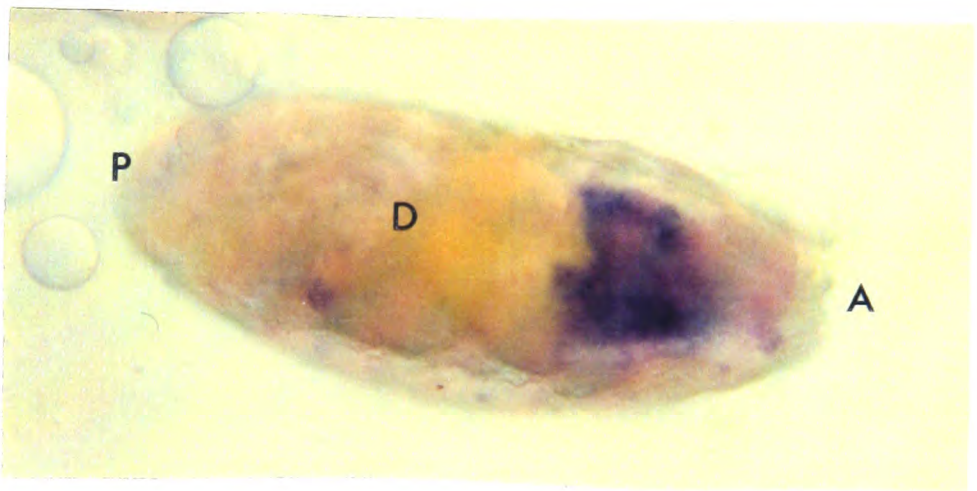
Stage 5.
Cell
formation.



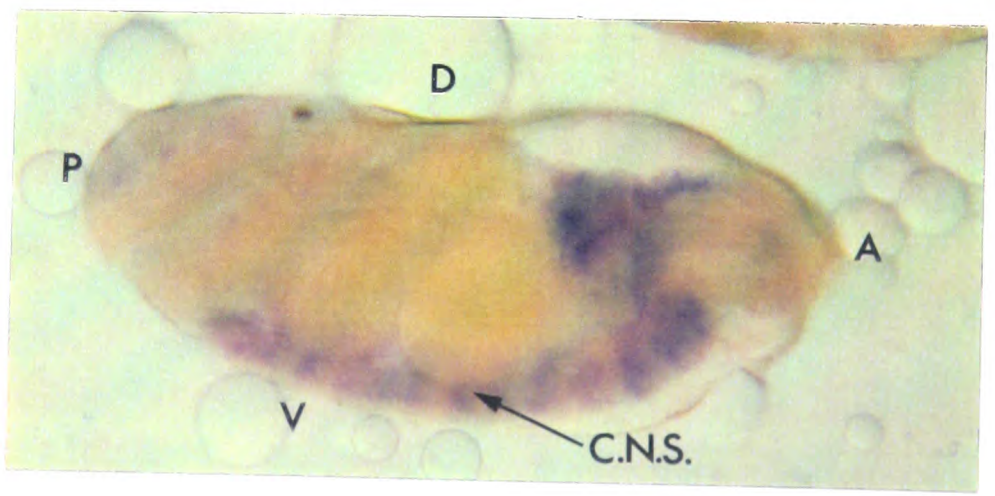
Stage 9.
Stomodeal plate
formation.



Stage 15.
Dorsal closure
complete.



Stage 16.
Condensation
of C.N.S.



A= anterior; P= posterior; D= dorsal; V= ventral.

Fig. 4.13 *Dhr6* RNA *in situ* hybridisation to wholemount OrR embryos.
Stages from Wieschaus and Nusslein-Volhard, 1986.

4.2.2.2 *UbcD4* transcript can be detected all over wholemount embryos:

The *UbcD4* transcript appears to follow the pattern of expression seen for other E2s, but at later stages the concentration within the CNS is not so strong; RNA can still be detected weakly all over the embryo (figure 4.14).

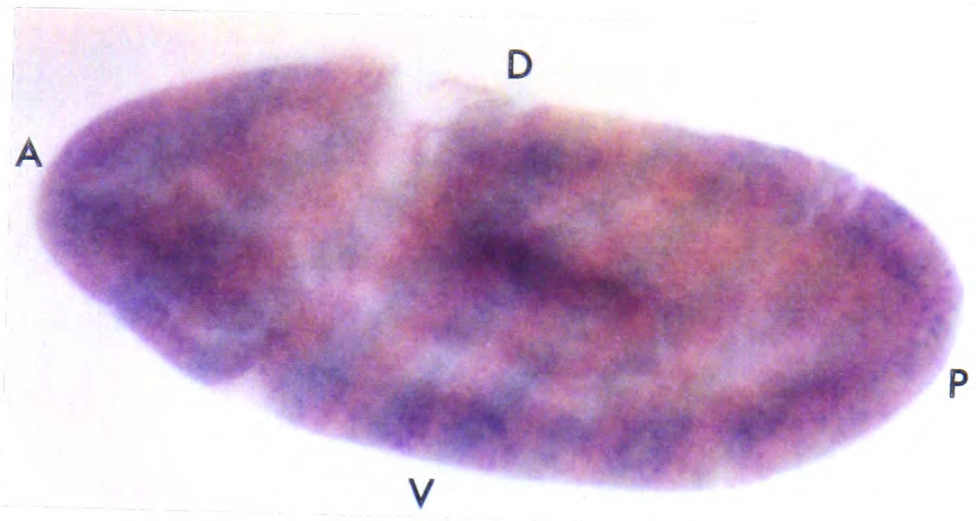
4.2.2.3 Discussion:

Transcripts from *Drosophila* E2 genes so far cloned all appear to undergo gradual restriction to the CNS during embryonic development, although this is less severe for *UbcD4*. This implies that the ubiquitin pathway has a role in nervous system development. The *ben* gene product may regulate nervous system development by axon growth cone guidance (Euk Oh *et al.*, 1994), and appears to be specific for certain neurons. Perhaps each of these E2 enzymes is responsible for a different set of neurons. *UbcD4* transcript can be detected throughout the embryo, reflecting its putative role as an E2 for bulk degradation of abnormal and short-lived proteins.

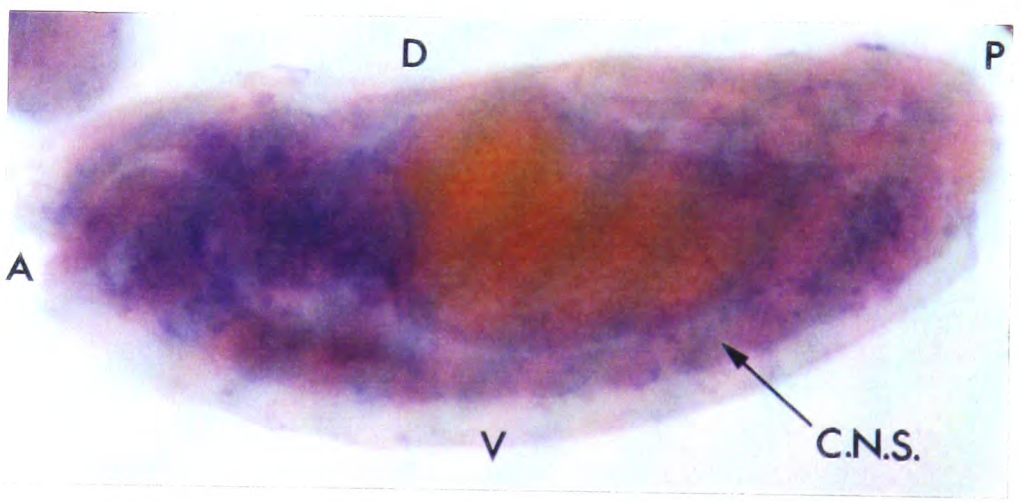
This study has only been carried out on five *Drosophila* E2 enzymes. There are probably many more, and others may show different patterns of tissue-regulated expression.

Stage 1.
Freshly laid egg.

Stage 9.
Stomodeal plate formation.



Stage 15.
Dorsal closure complete.



Stage 16.
Condensation of C.N.S.

A= anterior; P= posterior; D= dorsal; V= ventral.

Fig. 4.14 *UbcD4* RNA *in situ* hybridisation to wholemount OrR embryos.
Stages from Wieschaus and Nusslein-Volhard, 1986.

CHAPTER 5

MUTAGENESIS

5.1 INTRODUCTION:

The full biological significance of protein ubiquitination can only be revealed by a molecular genetic analysis in a multicellular organism. In order to directly determine the role of the ubiquitin pathway, and E2 enzymes in particular, in the *Drosophila* life-cycle, it is necessary to determine mutant phenotypes for each gene. This chapter dicusses methods employed to create mutants.

5.2 P ELEMENT MEDIATED MUTAGENESIS (a brief summary):

P transposable elements can create mutations when inserted within a gene (for review see Engels, 1989). Mobilization of P elements already present in the genome can result in novel P element insertions. This formed the basis of the first two methods used in the attempt to create mutant E2 enzymes.

5.2.1 "SITE SELECTED" P ELEMENT MUTAGENESIS:

This experiment was performed in collaboration with Dr P. Emery in Dr K. Kaiser's laboratory at Glasgow University. The method employed is used to try to create mutants where genes have been cloned, but associated phenotypes are unknown, and is based on Kaiser and Goodwin (1990). Flies were screened for insertions in *Dhr6*, *UbcD1*, *UbcD2*, *ben* and *uch-D* .

If a transposon lies within or close to any genetic locus this can be detected by PCR amplification between a primer specific for the transposon and one specific for the gene of interest. As PCR amplification is very sensitive, one fly with such a configuration can be detected in a large excess of flies.

The crosses shown in figure 5.1 were performed, and 10,000 mutagenized flies with possible new insertions were produced. By PCR on DNA from progeny embryos, a fly with a required insertion was sought by subdivision of cages of 100 flies and further rounds of PCR. Insertions both 5' and 3' to the genes were detectable due to using two primers for each gene (primers 16-28Y, A26 and A79), and the P element 31-mer terminal inverted repeat, present at both ends, as the P primer (P31). As many spurious bands were seen, gels were blotted and probed with a digoxygenin labelled probe from the gene of choice. Eventually single females, from cages of 10 identified to have possible required P insertions, were allowed to lay eggs in individual vials before PCR amplification was performed on single fly DNA.

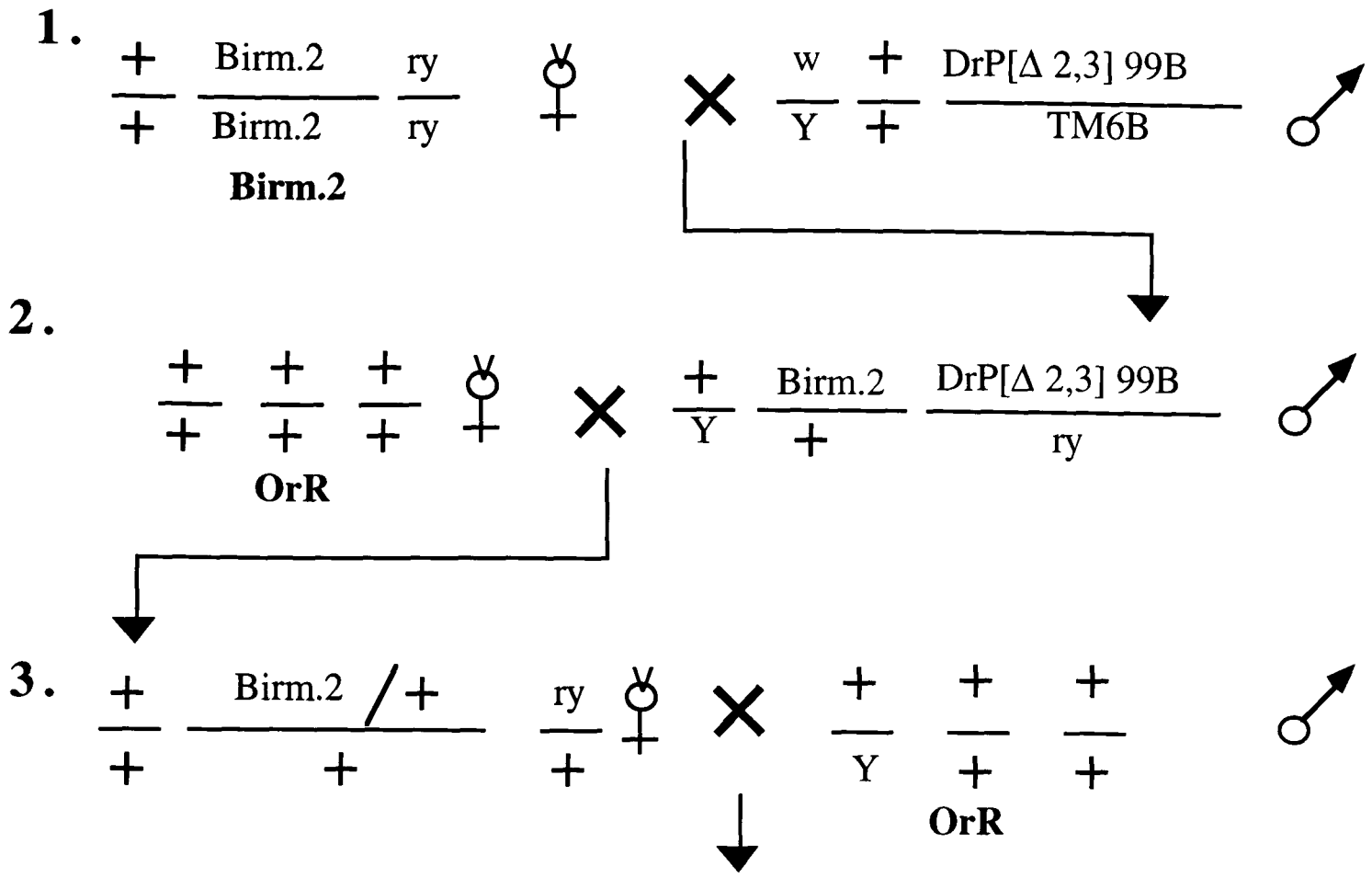


Fig. 5.1 Crosses for P element site selected mutagenesis.
Birmingham 2 (Birm.2) flies have 17 defective P elements on chromosome 2, non-mobile due to internal deletions. They still have intact terminal inverted repeats, and so can be mobilized when provided with a source of transposase from P[Δ 2,3] elements.

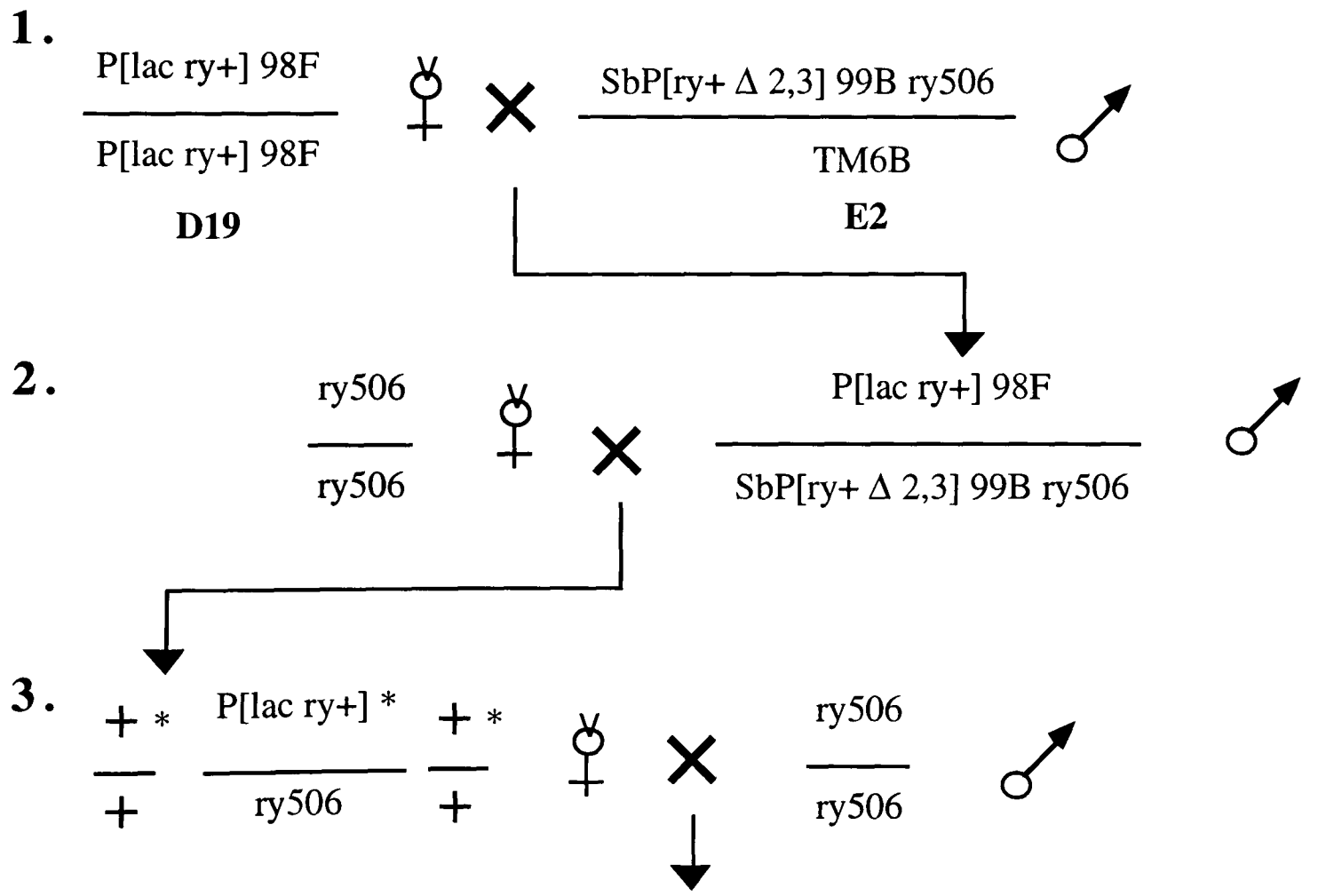


Fig. 5.2 Crosses for local jumping of the D19 P element into *uch-D*.
* = possible new insertions.

Transposition rates of P elements (Engels, 1989), showed 10,000 flies should have been enough for a P element to be inserted on average every 1kb along the *Drosophila* genome. However, despite several possible P insertions being followed to the single fly stage, no insertions for the genes screened were detected in the mutagenized flies. The *singed* gene has a site where P elements frequently insert, and this had originally been used by Dr P. Emery to show P elements were transposing. However, this was found to be due to the *singed* primer used giving "false positive" bands in PCR amplifications with the P31 primer. P elements were not transposing at expected levels. This was shown by a radioactive P element DNA probe hybridising to the same size bands for Birm.2 and "mutagenized" flies in genomic Southerns, and experiments performed by Dr. P. Emery, with a new primer, detecting only 1/5000 insertions in the *singed* gene.

5.2.2 LOCAL P ELEMENT JUMPING:

The efficiency of insertional mutagenesis can be significantly enhanced by using a P element located near the target of interest (Golic, 1994; Tower *et al.*, 1993; Zhang and Spradling, 1993). From figures in these publications for rates of insertion, it was calculated 80 new P element insertions, 2-128kb from the original P element, should be created in a population of 2,000 flies in a local jumping experiment. Experiments were designed to try to jump P elements into the genes of interest using elements known to reside nearby.

The first local jumping experiment performed used the single P element in D19 flies, known to be 10-15kb away from the *uch-D* gene (Zhang, 1992; Zhang *et al.*, 1993). The crosses used to jump this P element are shown in figure 5.2. Approximately 6,000 flies were screened for insertions in *uch-D* in the same way as for the "site selected" mutagenesis experiment. No insertions in *uch-D* were found, although control PCR amplifications showed the primers could amplify the correct regions of DNA, and genomic Southerns of single fly DNA extracts probed with ^{32}P-labelled P element DNA, showed P elements were transposing.

As no insertions were produced for *uch-D*, and many of the P element insertions near to other ubiquitin pathway genes resided up to 300kb away, beyond the zone of enhanced transposition, no further local jumping experiments were carried out.

5.3 MUTAGENESIS USING THE GAL4 ENHANCER TRAP SYSTEM:

5.3.1. INTRODUCTION:

P element mutageneses yielded no mutants, so another experiment was designed as a first step to determining the function of E2s in *Drosophila*. This involved altering the active site cysteine residue, and using the GAL4 enhancer trap system (Brand and Perrimon, 1993) as a means of expressing mutant E2s in a specific manner, to generate dominant phenotypes.

Expression of a dominant negative protein must be regulated as it may be lethal. The ability to restrict expression of the protein would be a useful means of analyzing its role in development, as if expressing the mutant protein everywhere was lethal, it might be more informative to view the effects of temporal or tissue specific expression. Driving expression of a gene from a heat shock promoter allows it to be turned on at a specific point in development by heat shocking the animal, but expression is ubiquitous. Also basal levels of expression are observed from heat shock promoters, and heat shock itself can induce phenocopies (Parkhurst and Ish-Horowitz, 1991). The use of tissue-specific promoters allows transcription to be restricted to a defined subset of cells, but this is limited by the availability of cloned and characterised promoters.

One way of avoiding the problems associated with heat shock or tissue specific promoters is to put the gene of interest under the control of a transcriptional activator in a different line of flies to those expressing the activator. Only when the lines are crossed is the target gene turned on in the progeny. Different lines can be made expressing the transcriptional activator in numerous specific patterns. Brand and Perrimon (1993) used the yeast GAL4 transcriptional activator in such a system, as GAL4 had been shown to be able to promote transcription in *Drosophila* (Fischer *et al.*, 1988), and bound an optimized binding site with high affinity (Webster *et al.*, 1988).

O'Kane and Gehring (1987) fused the *E.coli lacZ* gene to the weak P transposase promoter, and showed this reporter gene could respond to neighbouring transcriptional regulatory elements. This was developed as an "enhancer trap" system, to identify transcriptional regulatory elements in the *Drosophila* genome. Fusing the GAL4 coding sequence to the P transposase promoter in a transformation vector, enabled Brand and Perrimon (1993) to create "enhancer trap" lines. These direct expression of GAL4 in a wide range of patterns, depending on the genomic site of integration of the vector. By cloning the gene of interest behind a promoter

with five optimized GAL4 binding sites (the upstream activation sequence or UAS) on a P element transformation vector, and creating flies homozygous for this construct, lines are made where the gene can be expressed in a number of different patterns by crossing them to specific GAL4 enhancer trap lines (figure 5.3). To view the expression pattern of a particular GAL4 line, progeny from crossing it to a UAS *lac*Z line are stained for β-galactosidase expression.

The following section describes using GAL4 lines to overexpress mutant E2 enzymes.

5.3.2. RESULTS:

Dhr6 is the *Drosophila* RAD6 homologue, and can functionally substitute for RAD6 in its DNA repair and U.V. mutagenesis functions (Koken *et al.*, 1991a.). This implies it has the same functions in *Drosophila*, and may have an informative mutant phenotype. The *ben* mutant phenotype is known: the axon of the giant fibre neuron fails to form its terminal bend in the thoracic ganglion, and thus fails to connect normally to the tergal depressor of the trochanter motorneuron and *ben*$^-$ flies are unable to make the jump response (Thomas and Wyman, 1984). Mutating *ben* in the same manner as *Dhr6* therefore acts as a control for the whole process, as it can be seen if the giant fibre neuron is affected.

Changing the active site cysteine (Cys) residue of RAD6 to alanine or valine produced the same mutant phenotype as seen with *rad6* null alleles (Sung *et al.*, 1990), proving the active site cysteine residue of an E2 enzyme is essential. It was decided to mutate the active sites of *Dhr6* and *ben* to alanine (Ala) and serine (Ser). Alanine is the same as the cysteine residue minus the side chain, and serine is approximately the same size as cysteine, but with a hydroxyl group instead of a sulphydryl group. Sullivan and Vierstra (1993) showed a stable adduct was formed between ubiquitin and the ubiquitin-conjugating enzyme ATUBC1+ when its active site was mutated to Ser. Ubiquitin became attached to the E2, but could not be removed for conjugation to target proteins.

Overexpression of both mutant proteins should result in a mutant phenotype as the alanine mutant should recognise and bind to target proteins, but might be unable to conjugate to ubiquitin. Serine mutant proteins should bind to both ubiquitin and the target proteins, but ubiquitin might be bound irreversibly, and so might not be transferred.

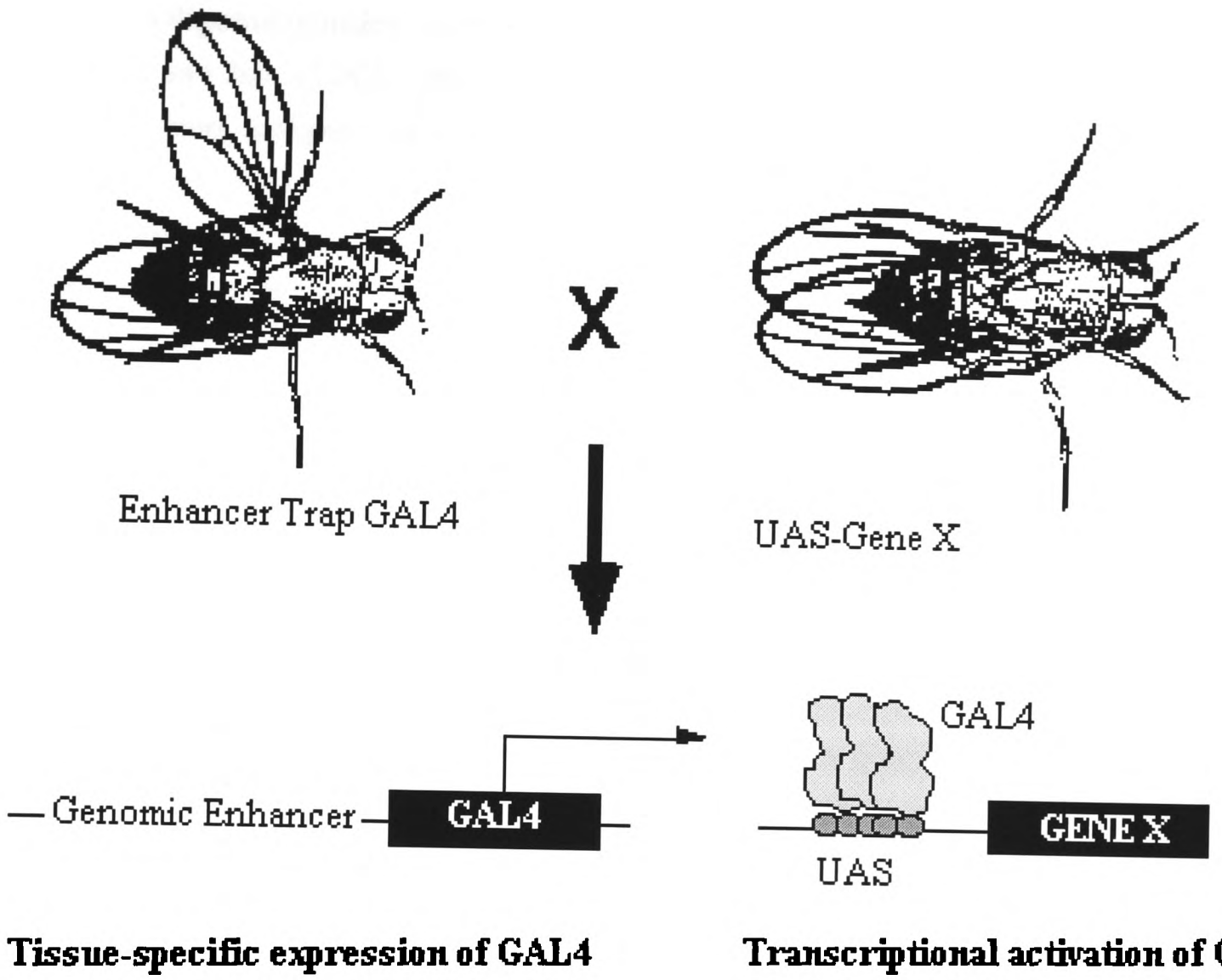


Fig. 5.3 The GAL4 enhancer trap system (after Brand and Perrimon, 1993).

5.3.2.1. Site directed mutagenesis of *Dhr6* and *ben* active site cysteine residues:

Oligonucleotides were designed for PCR amplification of the coding regions of*Dhr6* and *ben* cDNAs, and to alter their active sites to alanine and serine. Outer primers were designed with 5' restriction enzyme sites (*Eco*RI on the 5' primer, *Xho*I on the 3' primer), so they could be cloned easily, and two nucleotides upstream of the restriction enzyme site, so enzymes cut efficiently. One outer primer for each gene was located upstream of the translation start sequence and downstream of the transcription start, and the other was just upstream of the polyadenylation site. The middle two primers were complementary to each other, and contained the mutated codon. The active site cysteine residues of *Dhr6* and *ben* both have the codon TGC. The middle primers were designed to change this to GCC for alanine, the most common codon used in *Drosophila* for alanine, and AGC for serine.

Dr P. zur Lage carried out PCRs, as described in figure 5.4, with 25 cycles of 25sec at 94°C, 35sec at 50°C, 2.5min at 68°C. Primary PCRs were performed with template DNA of plasmid containing the cDNA for each gene, and secondary PCR amplification used 0.5µl of both primary PCRs per 100µl reaction. The wild-type coding regions of both genes were also amplified.

After PCR amplifications, Helena Thomaides, a Summer student in the laboratory, gel purified the fragments, digested them with *Xho*I and *Eco*RI and cloned them into pUAST (figure 5.5). This is the P element transformation vector, designed by Brand and Perrimon (1993), to direct GAL4-dependent transcription of a gene of choice. The coding sequence of the gene is subcloned into the polylinker, downstream of five, tandemly arranged, optimised GAL4 binding sites (figure 5.6). The plasmid also has P element ends, containing the inverted repeats where the transposase binds, which enable the piece of DNA between these ends to be moved into the *Drosophila* genome when P transposase is present. Genes cloned in this plasmid are transcribed from hsp70 promoter regions upstream of the polylinker, and are polyadenylated due to the presence of the SV40 polyadenylation site. The mini *white* gene is present to enable transformed flies, carrying an insertion of this construct, to be identified by eye colours which range from pale yellow through to wild-type brick red in a *white*$^-$ background.

The six constructs created (both *ben* and *Dhr6* with alanine, cysteine, or serine at the active site) had to be sequenced to show the active sites had been mutated, and no other mutations had been induced by *Taq* polymerase during the

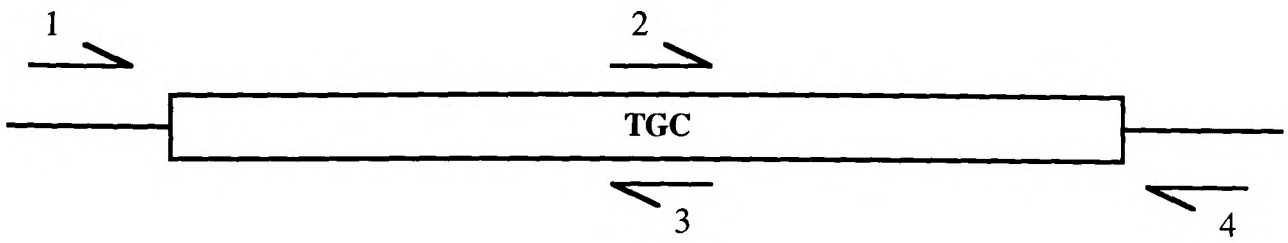


Fig. 5.4 PCR mutagenesis of the active site cysteine residue, shown in bold, of *Dhr6/ben.*
Dhr6 primers: 1 = D61; Ala2 = D62; Ala3 = D63; Ser2 = D6S2; Ser3 = D6S3; 4 = D64.
ben primers: 1 = D31; Ala2 = D32; Ala3 = D33; Ser2 = D3S2; Ser3 = D3S3; 4 = D34.
See Materials and Methods for oligonucleotide sequences.

Fig. 5.5 Plasmid map of pUAST.

PCR amplifications. The four primers used, for each gene, in the PCR amplifications were used for sequencing the constructs. The wild-type *ben* construct was found to have a mutation, so it was remade and sequenced. Oligonucleotides RK1 and RK2 were used to sequence the active site regions of *Dhr6* and *ben* respectively. The active sites were seen to be mutated as expected (figure 5.6) and no other mutations were found in the final pUAST constructs.

5.3.2.2 The creation of transformed lines:

P element mediated transformations were performed for all six constructs. The helper plasmid used was phs70Δ2-3wc, and in total, approximately 3,000 w^{1118} embryos were injected. Lines of the transformants produced were screened for P element insertions in Southern transfer experiments using ^{32}P-labelled 400bp *Sal*I and 838bp *Hind*III gel purified fragments from pπ25.7wc, to hybridise to *Eco*RI digested genomic DNA. As expected, two bands hybridised to the probe in each case (see figure 5.10 for genomic Southerns of the *Dhr6* transformed lines utilised in further experiments) as *Eco*RI should cut once within the insertion (see figure 5.8). Different lines are necessary for each construct, in order to show any effects are due to the construct, not the position of insertion. The number of different homozygous transformed lines produced is shown in table 5.1.

Only one line was obtained that had been transformed with *Dhr6* Cys. This insertion was seen to be on the Y chromosome as no coloured eyed females were produced after sibling crosses. The eye colour of these male flies was pale yellow implying that the *white* gene was not being efficiently expressed, and this was presumably also true of *Dhr6* . As it is necessary to have lines overexpressing wild-type DHR6 protein, in order to show that any phenotypes from overexpression of a mutant protein are due to the mutation itself, not just to overexpression of the protein *per se*, more *Dhr6* Cys lines were necessary. An experiment was designed to jump the P element present on the Y chromosome in *Dhr6* Cys line1, to different sites in the genome by exposing it to P element transposase as shown in figure 5.7. New insertions could be easily detected, as coloured-eyed females must carry new insertions.

The crossing scheme of figure 5.7 was designed so as to give secondary insertions in the same genetic background as primary transformants, so there could be no effects due to constructs being overexpressed in different genetic backgrounds. At first all crosses, except 5b. and 6b. (figure 5.7), were carried out. The Δ2-3

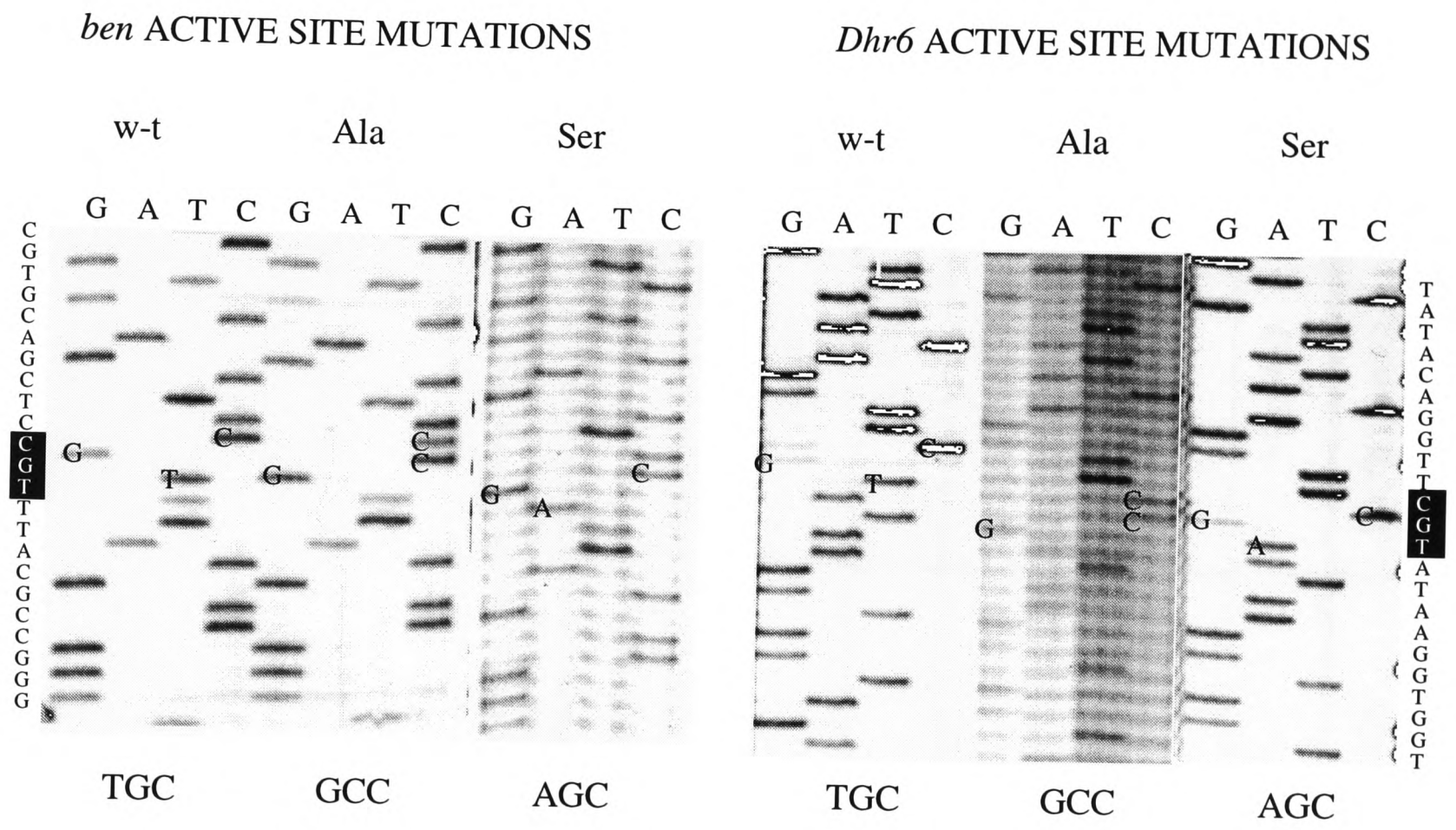


Fig. 5.6 Sequence of active site regions of *Dhr6* and *ben* pUAST constructs.

CONSTRUCT	NO. LINES
Dhr6 Cys	1 (on Y)
Dhr6 Ala	4
Dhr6 Ser	5
ben Cys	9
ben Ala	0
ben Ser	6

Table 5.1 Number of transformed lines with different insertions from injection of pUAST constructs.
Dhr6/*ben* Cys = lines transformed with wild type constructs
Dhr6/*ben* Ala = lines transformed with Ala active site constructs
Dhr6/*ben* Ser = lines transformed with Ser active site constructs

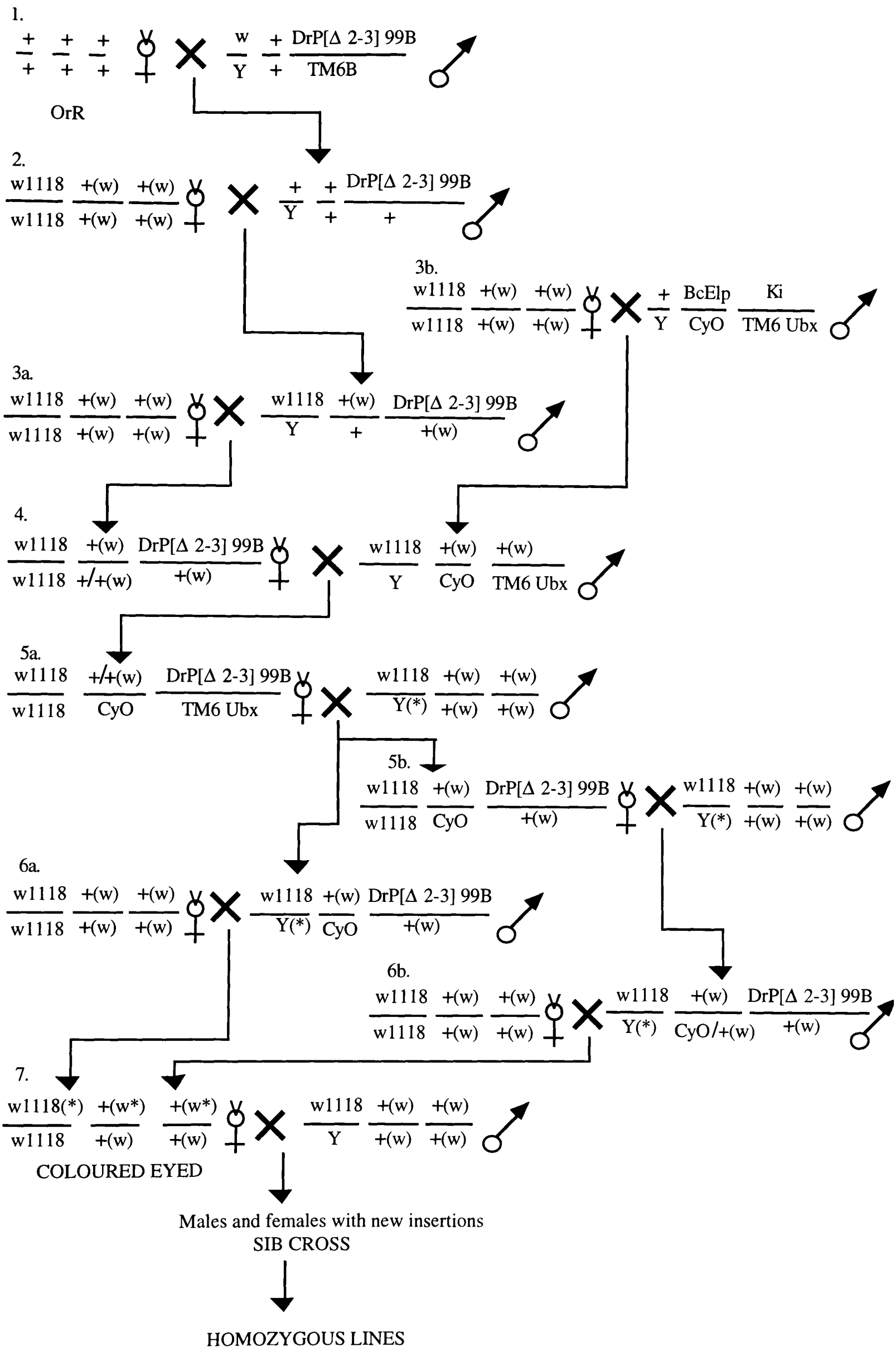


Figure 5.7 Crosses performed to mobilize the *Dhr6* Cys line1 insertion from the Y chromosome.
* = insertion, or possible new insertions; +(w) implies w-t chromosome, but from w1118 flies.
OrR= OregonR; Dr= drop; Bc= black cells; Elp= ellipse; Ki= kinked; Ubx= ultra bithorax; CyO= curly oster.

construct provided transposase for jumping the *Dhr6* Cys insertion. Females from cross 6b. could have had new insertions; 386 were screened, but all had white eyes. Transposition was shown to have been occuring, by probing a single fly genomic Southern of extracts from males from cross 6a. with a P element probe. New insertions were observed (results not shown).

As transposition had been taking place, more females with possible new insertions were created by crosses 5b. and 6b.. Six females with coloured eyes were produced from these crosses. Two coloured eyed females and one red eyed male were also seen in the progeny of cross 5b., implying that they had new insertions. This should be impossible for the females, as the Δ2-3 and Y chromosome P insertion had never been present in the same fly. However, the male could have Δ2-3 and the Y insertion, and his red eyes could be produced by somatic transposition of the P element. Alternatively all three flies could have been produced by a single non-disjunction event creating a female with both the Y chromosome or part of the Y chromosome, and therefore the P insertion, and the Δ2-3 element.

To show the nine lines created had new insertions, genomic Southerns were probed with the same P element probe as before. Genomic DNA was digested with *Eco*RI which cuts once in the insertion (figure 5.8), so again two bands were produced for each insertion, corresponding to each P element end. Each insertion gives bands of different lengths according to the position of the flanking *Eco*RI sites. From the Southern it appeared *Dhr6*Cys line 2 has three insertions, *Dhr6*Cys lines 3, 5, 6, 7 and 10 have two insertions, and *Dhr6*Cys lines 4 and 9 have one (figure 5.9). However, one weak band is present in six of the lines and corresponds in length to the smaller band produced from the *Dhr6*Cys line 1 Y chromosome insertion (figure 5.10). Each lane of the genomic Southern contains DNA extracted from five males and five females, so the weak band could correspond to the Y chromosome P element in male flies, which has not been excised during transposition. The larger band for this insertion may be obscured by bands present in males and females which are more prevalent, so appear to hybridise more strongly to the probe. If this is true then *Dhr6*Cys lines 2, 5, and 6 may have two new insertions, and 3, 4, 7, 8, 9, and 10 may have one new insertion. This could have been tested by comparing male and female fly DNA hybridisation patterns to the P-element probe.

It was necessary to prove these P element bands did come from the *Dhr6* construct insertions, so the filter was stripped and reprobed with ^{32}P-labelled *Dhr6* cDNA. *Dhr6* is expected to hybridise to one band per insertion (figure 5.8), and also to a band corresponding to the genomic copy of the gene as *Eco*RI does not cut

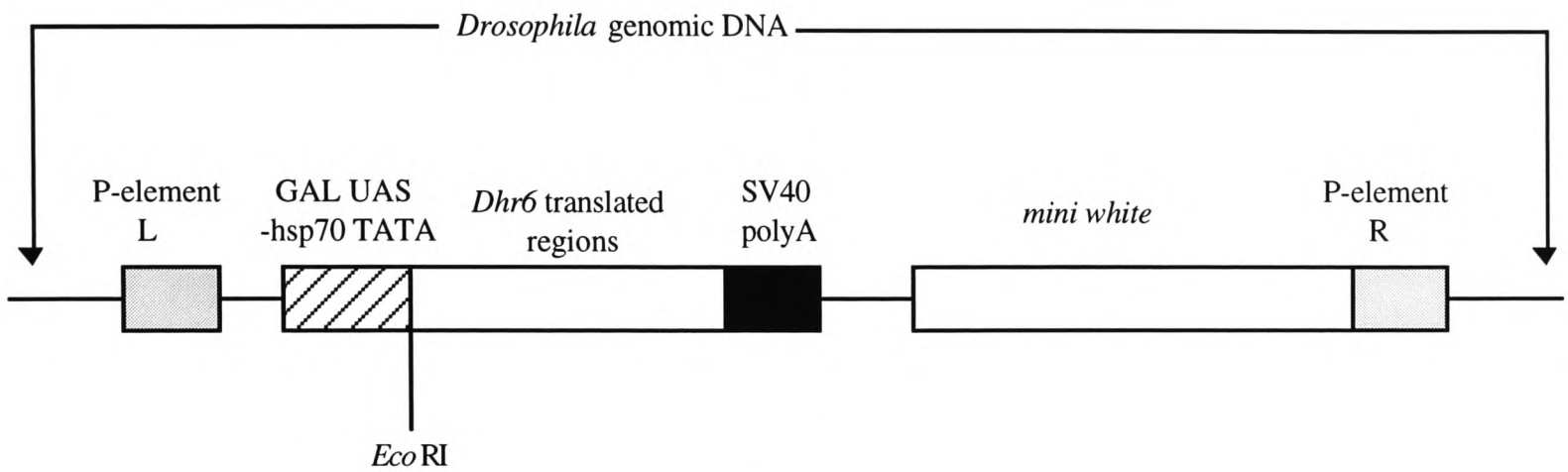


Fig. 5.8 Insertions of *Dhr6* from pUAST constructs

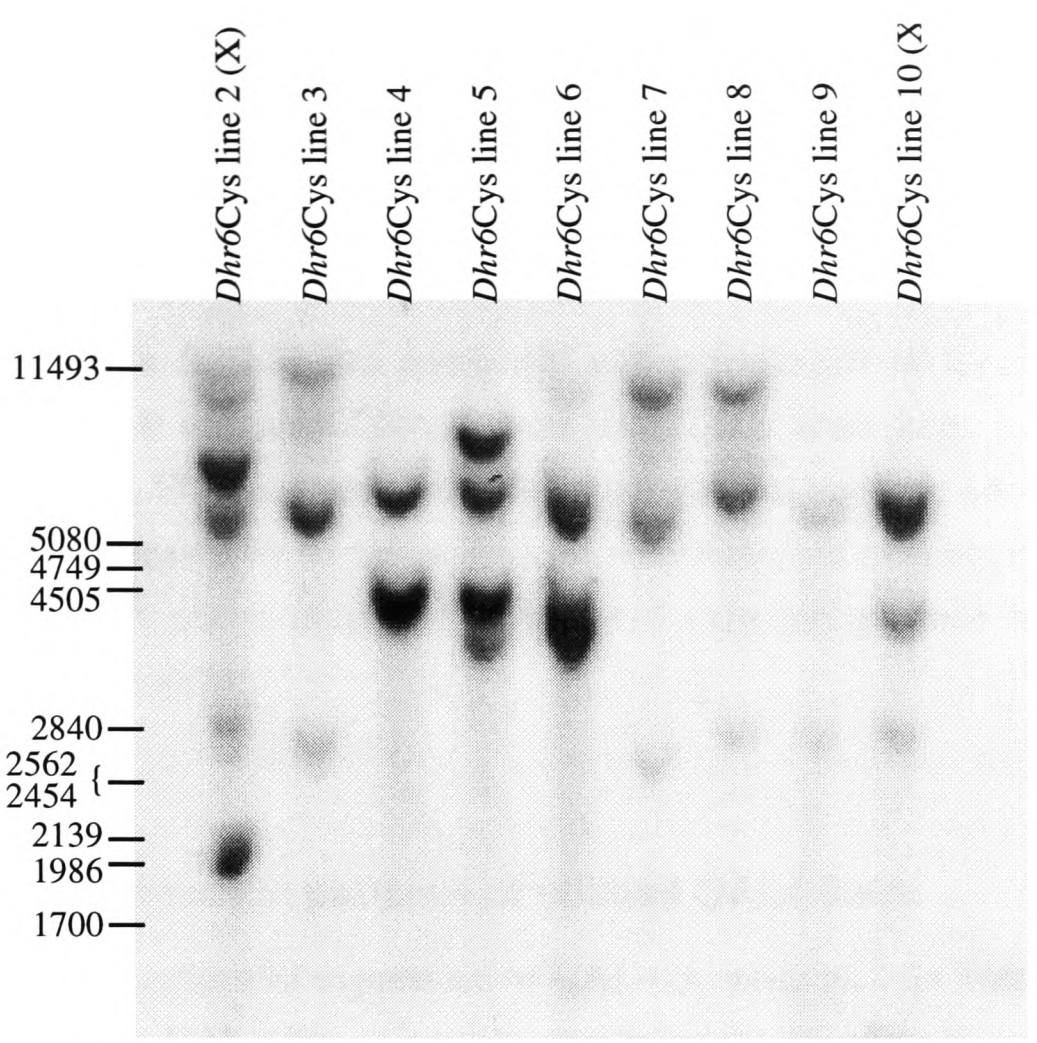


Fig. 5.9 Genomic Southern to show P element insertions of lines produced by transposition of *Dhr6*Cys line 1 (Y) P element.
Bacteriophage lambda DNA digested with *Pst*I was used as a size marker.

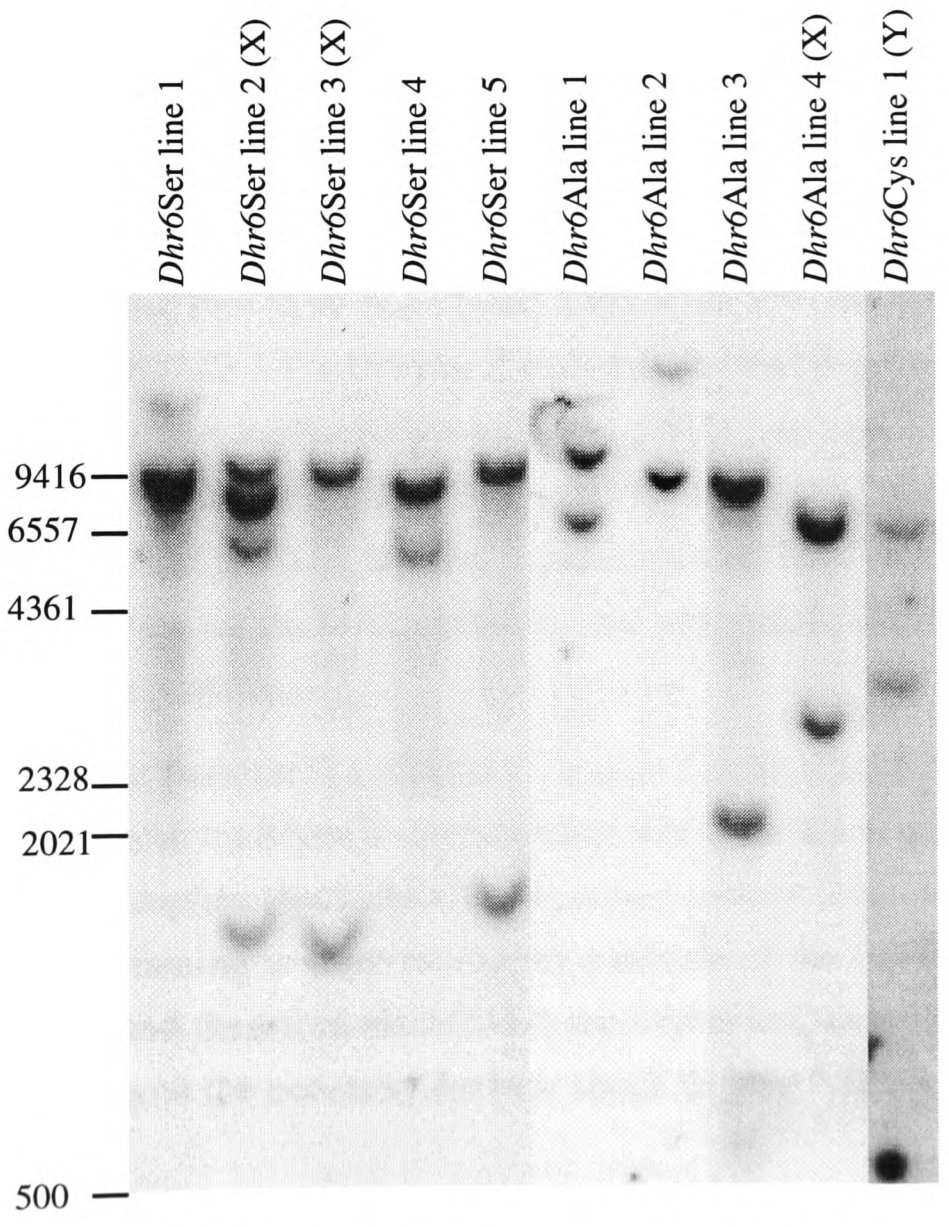


Fig. 5.10 Genomic Southern to show P element insertions of *Dhr6* lines produced by transformation.
Bacteriophage lambda DNA digested with *Hind*III was used as a size marker.

within the gene. One band per insertion was seen to match when the autoradiographs were compared (results not shown), implying all lines do contain the *Dhr6* Cys construct in different copy numbers and positions.

Figure 5.10 shows combined autoradiographs of Southerns probed with radioactive P element DNA for all the *Dhr6* lines kept from transformation experiments. The Southerns from *ben* transformed lines are not shown. No *ben* Ala lines were created by transformation experiments, but as there were many Ser lines (table 5.1) to act as controls for the *Dhr6* experiment, these were not considered absolutely essential.

5.3.2.3 Expression patterns of utilised GAL4 lines:

The pattern of expression of GAL4 in enhancer trap lines can be detected by crossing each GAL4 line to a strain containing the *E.coli lacZ* gene under the control of the GAL4 upstream activating sequence (UAS*lacZ* flies) and staining the progeny for β-galactosidase expression. This was carried out, for embryonic stages, for all GAL4 lines used in later experiments:- heat shock (h.s.) GAL4 line, C22C and Krüppel (Kr) 5Y. Before staining, h.s.GAL4 x UAS*lacZ* embryos were collected on apple juice agar plates, and heat shocked at 39°C on a heating block for 30min. UAS*lacZ* embryos were also stained as a control.

No β-galactosidase expression was detected in UAS*lacZ* embryos (figure 5.11A.), implying that there is no basal level of *lacZ* transcription in these flies. The positive control of *Kr* 5Y x UAS*lacZ* embryos showed β-galactosidase expression in a broad band across early embryos (figure 5.11B.) as expected (Hoshizaki, 1994). The *Kr* gene product is expressed in a narrower band, and presumably this is how the GAL4 protein is expressed, but the β-galactosidase band is larger due to activation of *lacZ* expression by the GAL4 protein, and sequential diffusion of both GAL4 and β-galactosidase proteins.

Embryos from the h.s. GAL4 x UAS*lacZ* cross stain for β-galactosidase in all tissues throughout embryonic development when embryos are heat shocked (figure 5.12A.). This implies that GAL4 is expressed everywhere. A similar pattern of β-galactosidase staining is seen in progeny embryos of the C22C x UAS*lacZ* cross; β-galactosidase and therefore also GAL4 are expressed throughout embryogenesis in all tissues, without the necessity for heat shock (figure 5.12B.).

A.

B.

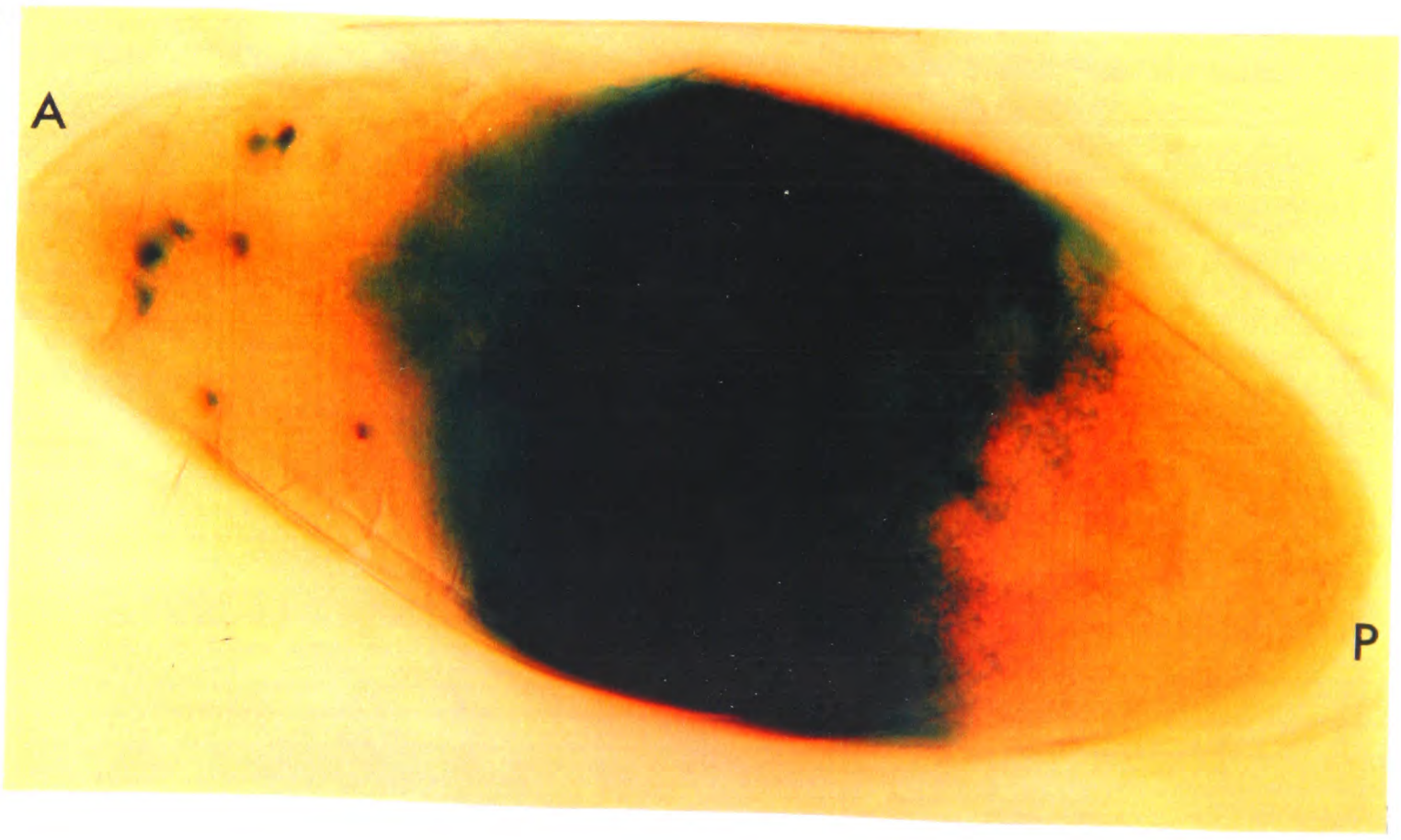
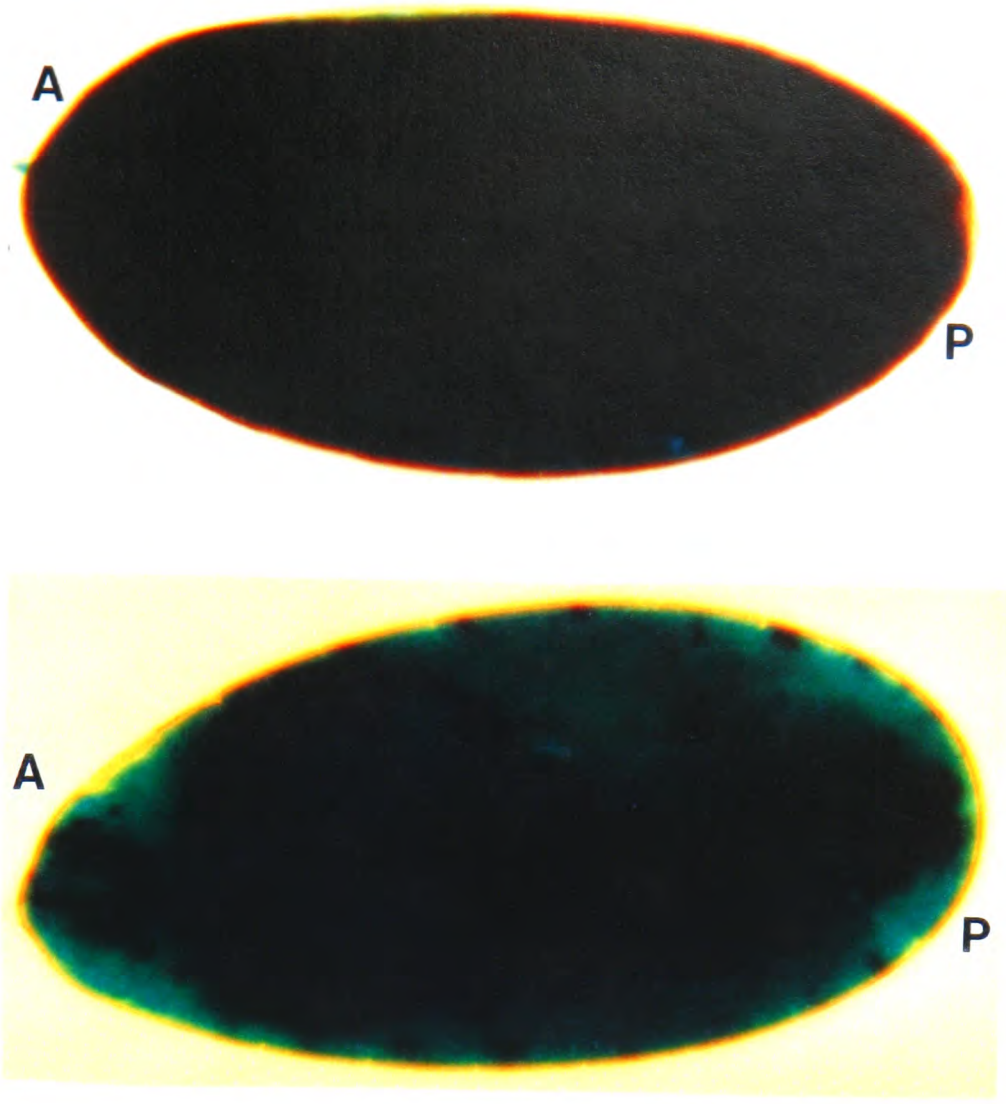
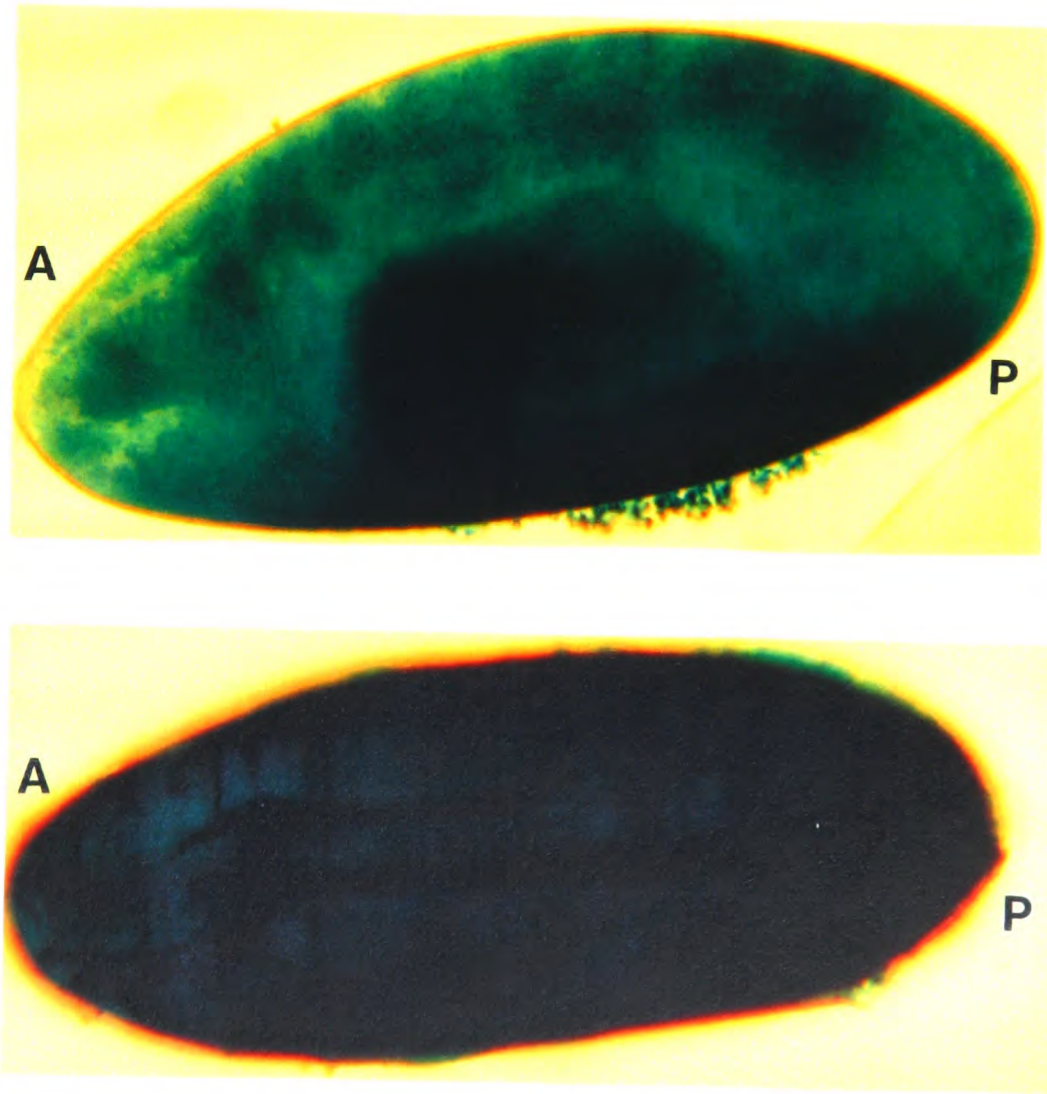


Fig. 5.11 Expression patterns of GAL4 lines.
Embryos were stained for β-galactosidase after crossing to UAS*lac*Z.
A. UAS *lac*Z control (stage 7: midgut invagination).
B. *Kr* 5Y (stage 3: pole cell formation.
A= anterior; P= posterior.

A.



B.



Embryos were stained for β-galactosidase activity after crossing to UAS*lac*Z.
A. h.s. GAL4 (top= stage 3: pole cell formation; bottom= stage 16: condensation of C.N.S.).
B. C22C (top= stage 9: stomodeal plate formation; bottom= stage 16: condensation of C.N.S.).

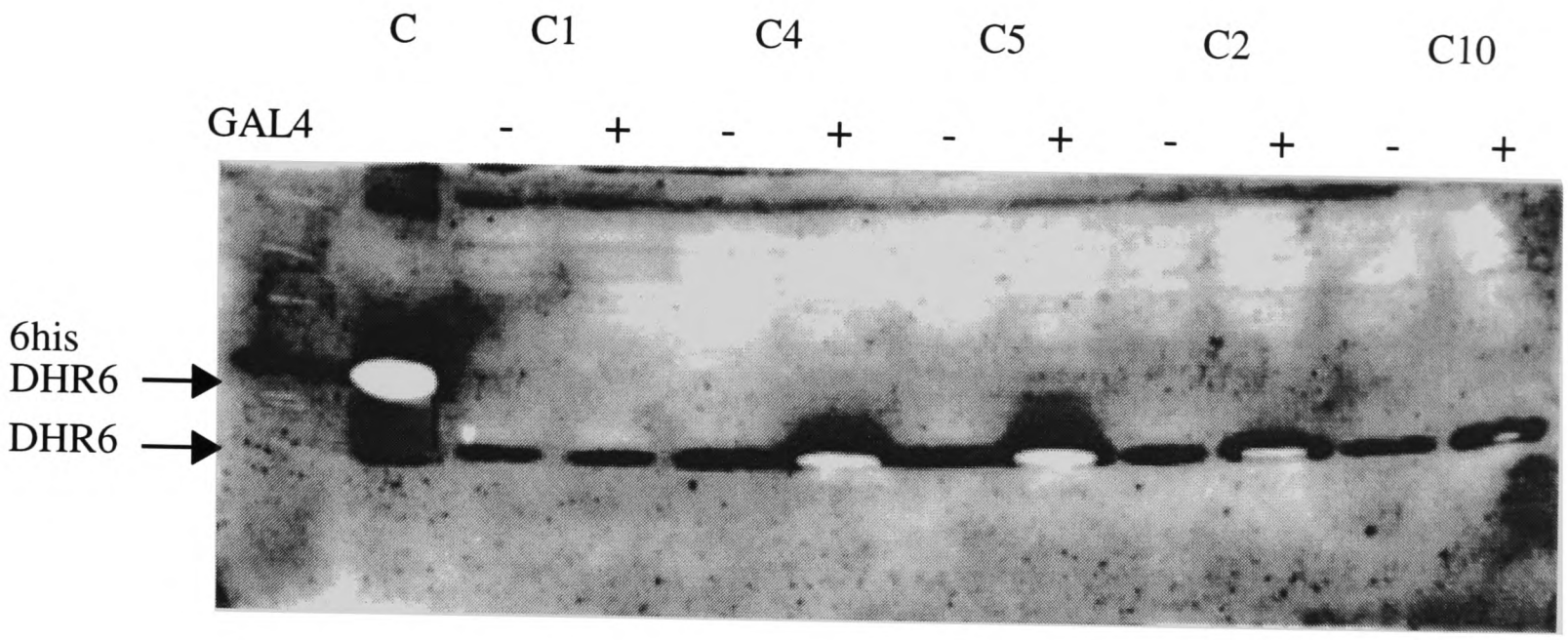
5.3.2.4 Transformed *Dhr6* lines overexpress DHR6 protein:

To determine whether protein was overexpressed from the transgenes in transformed lines, proteins were induced and levels of DHR6 protein were followed using a polyclonal DHR6 antibody raised against a GST fusion protein.

To induce *Dhr6* expression, all the *Dhr6* lines were crossed to the h.s. GAL4 line at 18°C to minimise basal expression from the heat shock promoter, and adult progeny were heat shocked at 39°C for 30 mins. In order to allow time for expression of both the GAL4 transcriptional activator, and the DHR6 protein under GAL4 control, flies were left to recover at room temperature for 3 hours. The h.s. GAL4 insertion is on the third chromomsome and is balanced by TM3 with *Stubble* (*Sb*) as the dominant marker. Both Sb^+ and Sb^- flies were kept frozen at -70°C, until protein extracts were made by Dr P. zur Lage.

Dr P. zur Lage ran total protein extracts on SDS PAGE gels with approximately 110μg protein loaded per lane, and DHR6 was detected on Western blots by a polyclonal DHR6 antibody (at a dilution of 1/1000), made in rabbits. The results of detection of the primary antibody by a secondary antibody and chemiluminescence (Dr P. zur Lage; see Materials and Methods) are shown in figures 5.13 and 5.14. Overexpressed bands of the correct size of 16.6kDa can be seen for all lines except the *Dhr6* Cys Y insertion (line1), and *Dhr6* Ala line 2. Comparing Sb^+ flies where GAL4 is being expressed, and Sb^- flies where there is no GAL4, shows there is overexpression of the protein in the presence of GAL4. In Sb^- flies a much weaker band is visible from expression of the genomic copy of *Dhr6*.

The DHR6 antibody binds to two proteins with different molecular weights in Ser lines. The lower band is the same size as the band for the Ala lines, and presumably corresponds to the overexpressed mutant DHR6 protein. When an E2 enzyme has Ser at the active site, ubiquitin can become irreversibly bound (Sullivan and Vierstra, 1993), so the higher molecular weight band could be the E2 bound to ubiquitin. In order to test this hypothesis, Dr P. zur Lage ran the same samples on two gels and performed Westerns using the DHR6 antibody for one gel, and a polyclonal ubiquitin antibody (Novocastra Laboratories Ltd., at a 1/500 dilution) to detect ubiquitinated proteins on the other gel. The results are shown in figure 5.15. The DHR6 antibody bound to the same overexpressed bands for the Ala and Ser lines as before, but only the higher molecular weight overexpressed band in Ser lines was bound by the ubiquitin antibody. It therefore appears DHR6 with Ser at the active site does bind to ubiquitin for a long period of time, and the interaction may be irreversible.



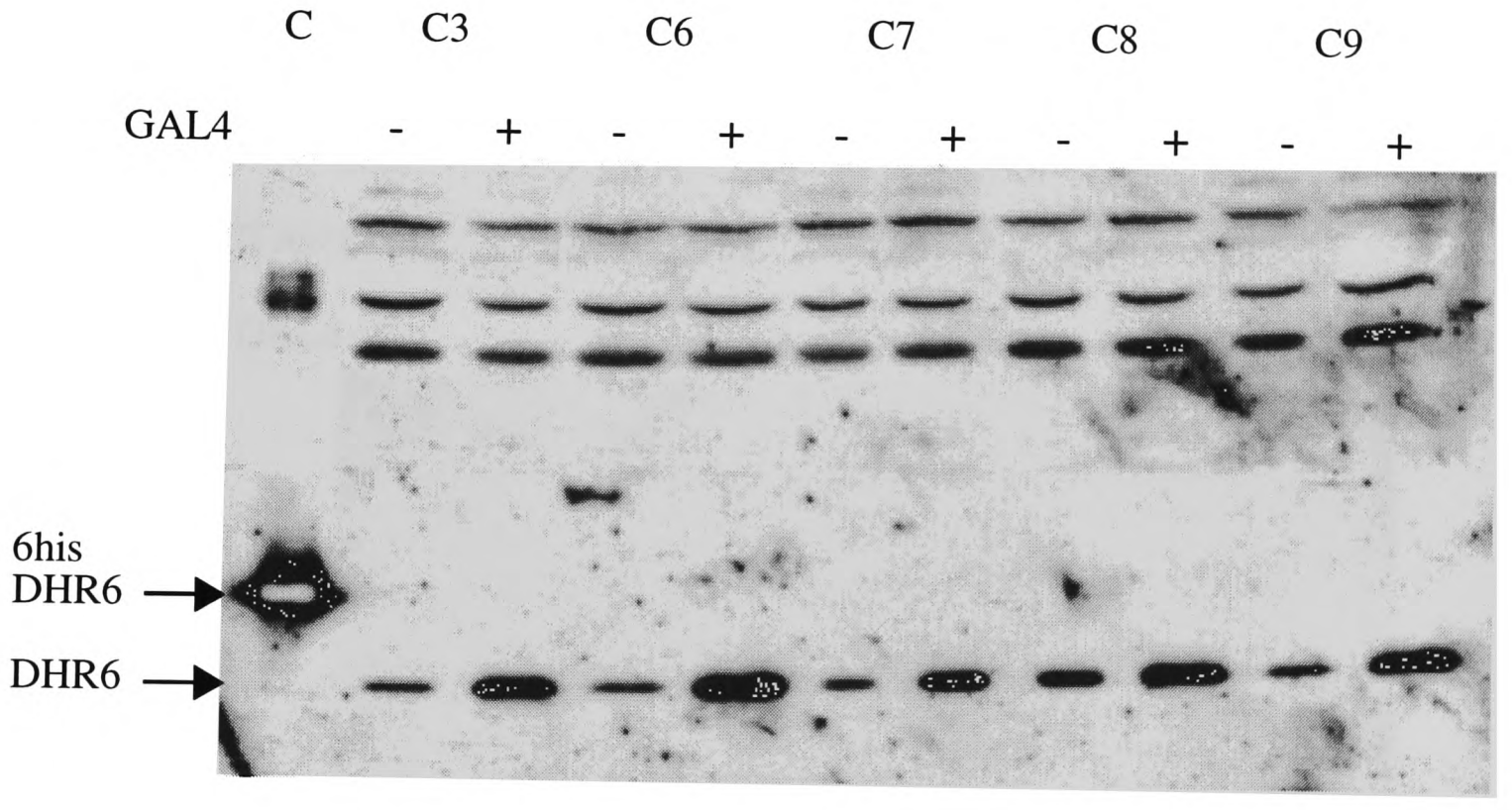


Fig. 5.13 Reaction of anti-DHR6 antiserum with extracts of transformed flies from *Dhr6*Cys lines in the presence or absence of GAL4.

Approximately 110µg protein were loaded per lane. The anti-DHR6 antibody was used at a 1/1000 dilution.

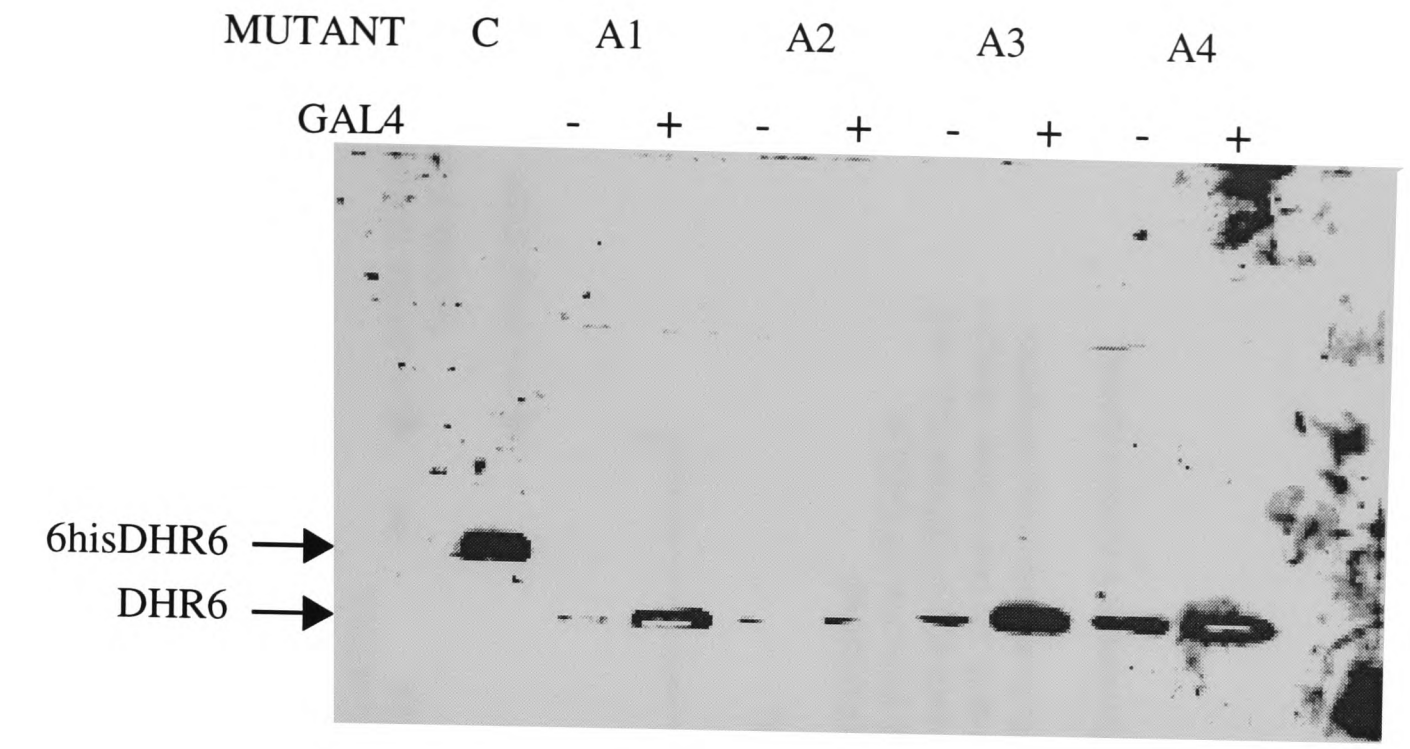
C = 6his DHR6 control (DHR6 protein expressed with a 6 his tag to allow purification on a nickel column).

Higher molecular weight bands present in the lower picture were also detected by preimmune serum.

C1-C10 = *Dhr6*Cys lines 1-10.

+ GAL4 = Sb^{+} flies; - GAL4 = Sb^{-} flies.

A.



B.

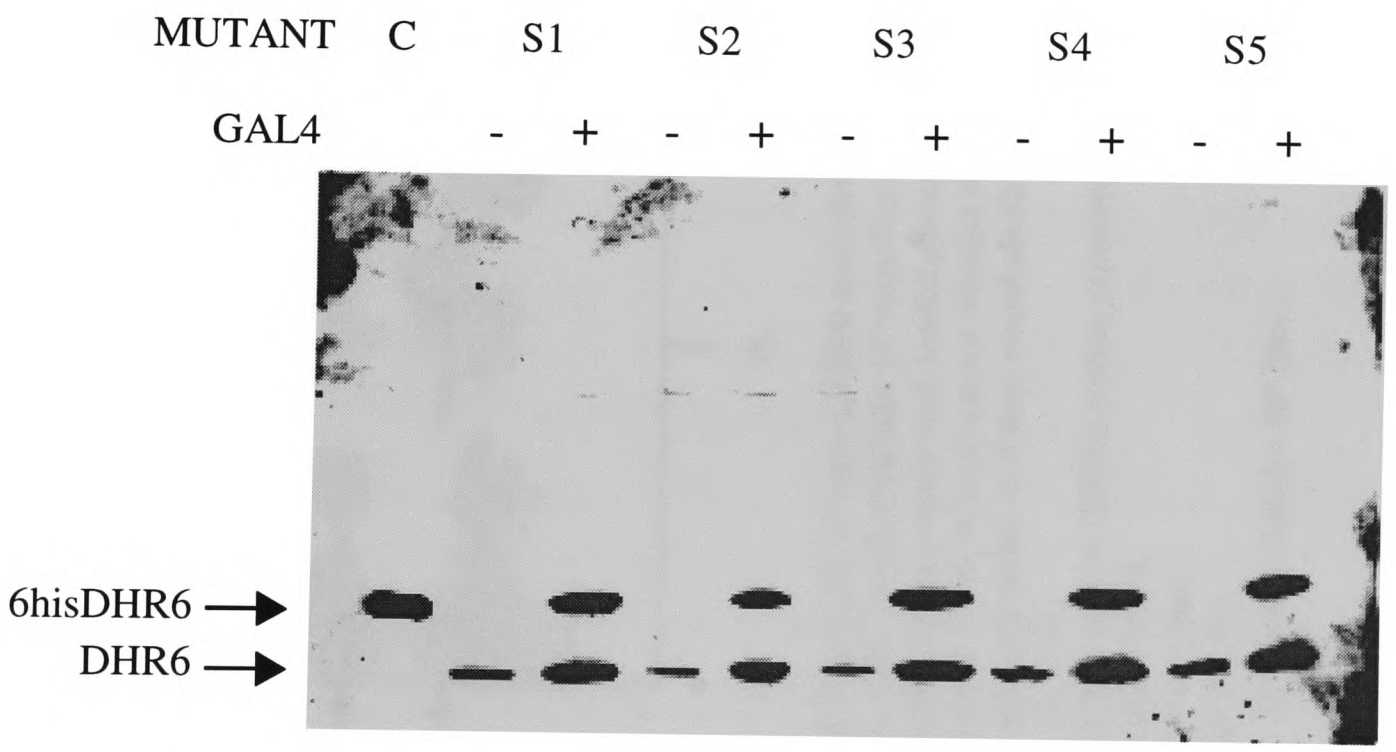


Fig. 5.14 Reaction of anti-DHR6 antiserum with extracts of transformed flies in the presence or absence of GAL4.
A. *Dhr6*Ala lines.
B. *Dhr6*Ser lines.
Approximately 110µg protein were loaded per lane. The anti-DHR6 antibody was used at a 1/1000 dilution.
C = 6his DHR6 control (DHR6 protein expressed with a 6 his tag to allow purification on a nickel column).
A1-A4 = *Dhr6*Ala lines 1-4.
S1-S5 = *Dhr6*Ser lines 1-5.
+ GAL4 = Sb^+ flies; - GAL4 = Sb^- flies.

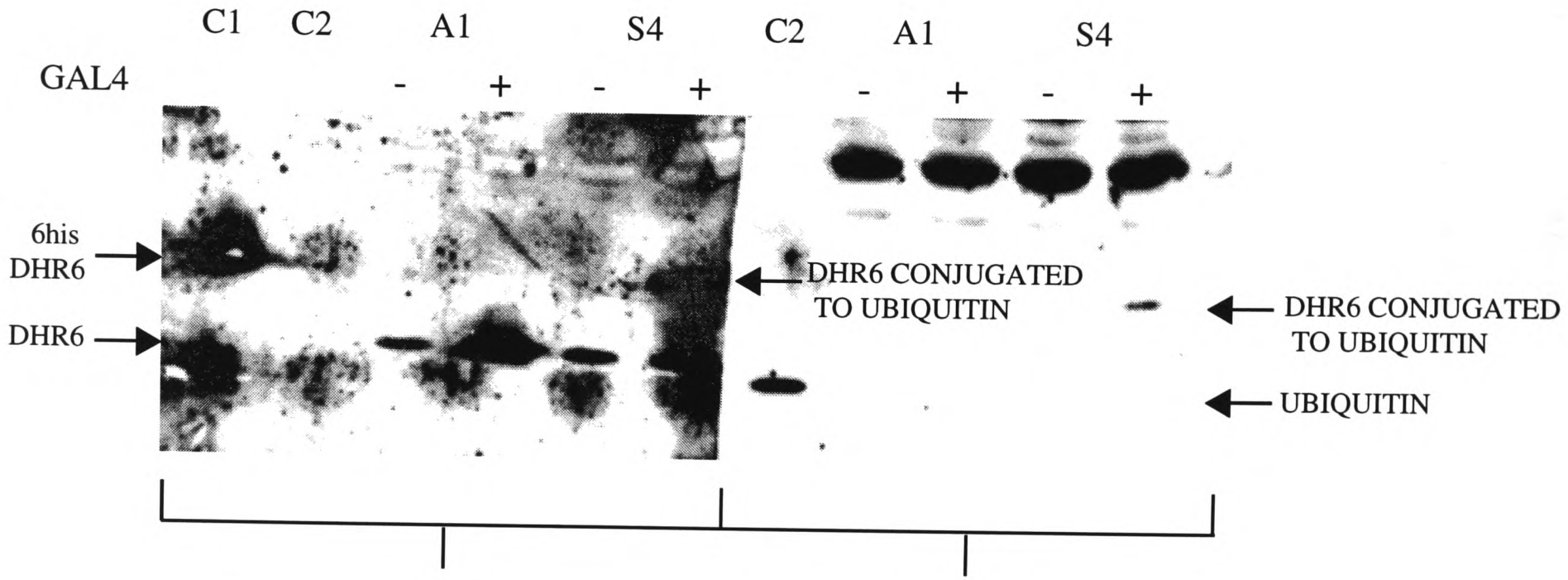


Fig. 5.15 Reaction of anti-DHR6 antiserum (A.) and anti-ubiquitin polyclonal antibodies (B.) with extracts of transformed flies in the presence and absence of GAL4.
Approximately 110µg protein were loaded per lane. The anti-DHR6 antibody was used at a 1/1000 dilution, and anti-ubiquitin at 1/500. It is not known whether the higher molecular weight bands in B. are due to large proteins stably bound to ubiquitin, or if they would also be detected using preimmune serum, as a commercial ubiquitin antibody was used.
C1 = 6hisDHR6 control (DHR6 protein expressed with a six his tag to allow purification on a nickel column).
C2 = ubiquitin control.
A1 = *Dhr6*Ala 1 line.
S4 = *Dhr6*Ser 4 line.
+ GAL4 = Sb^{+} flies; - GAL4 = Sb^{-} flies.

5.3.2.5 BEN protein was not overexpressed in the transformed lines:

To detect overexpression of BEN protein, all *ben* transformed lines were crossed to the h.s. GAL4 line, and treated as described above for detection of DHR6 protein in *Dhr6* lines. Dr P. zur Lage performed Western blots of protein extracts from these flies, but was unable to detect overexpression of the protein (results not shown).

It is not known why the *ben* lines did not overexpress BEN protein, but experiments with them were discontinued.

5.3.2.6 Overexpressing mutant DHR6 in the pattern of GAL4 line C22C, is lethal:

In order to investigate the effects of overexpressing mutant DHR6, lines expressing GAL4 in different patterns were used. The C22C GAL4 line expresses in all tissues throughout embryonic develoment (figure 5.12B.). The expression patterns after embryogenesis are unknown, but this line was used to show the effect of high level expression of DHR6 active site mutants throughout the embryo.

The P element GAL4 insertion in C22C is on the second chromosome, and is balanced by the SM5 chromosome which carries the dominant marker *curly* (*Cy*). When transformed *Dhr6* lines were crossed to C22C, a 1:1 ratio of Cy^- to Cy^+ flies was expected if overexpression of mutant DHR6 was not lethal (figure 5.16). Any Cy^+ flies produced could be examined for phenotypes due to overexpression of mutant DHR6 protein. The results are shown in table 5.2, and the method for statistical analysis on this data is described in Appendix II.

When w^{1118} was crossed to C22C, there was no significant difference in the ratio of Cy^- to Cy^+ progeny between males and females, or from the expected 1:1 ratio of total numbers of Cy^-:Cy^+ flies. The same was true for the cross to *Dhr6*Ala line 2 where there was no overexpression of mutant protein. There was no significant difference between the ratio of Cy^- to Cy^+ progeny from crosses to w^{1118} and the two transformed lines not overexpressing DHR6 protein (*Dhr6*Ala line 2 and *Dhr6*Cys line 1), although the progeny of the *Dhr6*Cys line1 cross contained significantly more Cy^+ than Cy^- flies. This was also true when the results for all three lines were pooled. The Cy^- flies from C22C crosses where there is no overexpression of DHR6 may be less viable than the Cy^+ flies, simply due to the presence of the mutant *curly* gene.

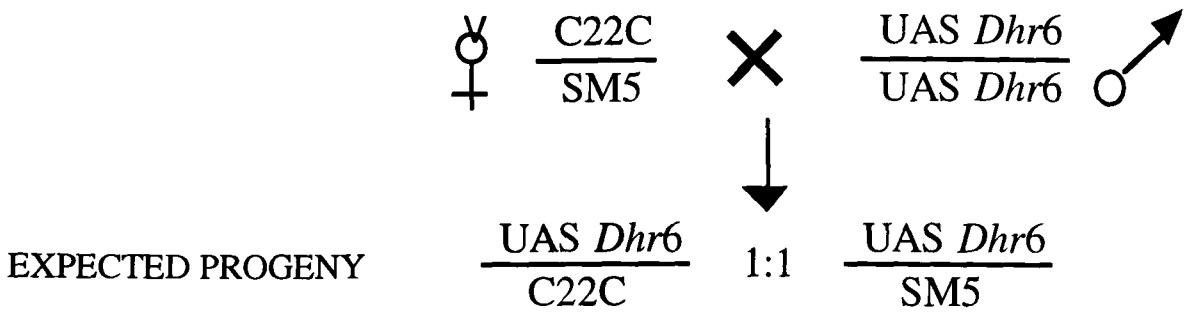


Figure 5.16 C22C crosses to UAS *Dhr6* transformed lines.

Strain	Protein Expression	C22C			
		Cy -		Cy +	
		Females	Males	Females	Males
w1118	na	56	57	65	73
*Dhr6*Cys1 (Y)	No	37	35	51	53
*Dhr6*Cys2 (X)	Yes	43	36	46	35
*Dhr6*Cys3	Yes	66	39	8	36
*Dhr6*Cys4	Yes	53	41	53	50
*Dhr6*Cys5	Yes	64	53	57	58
*Dhr6*Cys6	Yes	44	38	55	30
*Dhr6*Cys7	Yes	43	38	68	49
*Dhr6*Cys8	Yes	66	46	63	49
*Dhr6*Cys9	Yes	55	58	0	62
*Dhr6*Cys10 (X)	Yes	47	52	58	61
*Dhr6*Ala1	Yes	65	59	0	0
*Dhr6*Ala2	No	19	30	24	38
*Dhr6*Ala3	Yes	23	32	0	0
*Dhr6*Ala4 (X)	Yes	53	37	0	52
*Dhr6*Ser1	Yes	70	93	0	0
*Dhr6*Ser2 (X)	Yes	80	69	0	57
*Dhr6*Ser3 (X)	Yes	58	69	0	73
*Dhr6*Ser4	Yes	107	152	0	0
*Dhr6*Ser5	Yes	57	58	0	0

Table 5.2 Results of C22C crosses to *Dhr6* transformed lines.

There was no significant difference between the ratios of Cy^-:Cy^+ progeny from crosses of C22C to the two *Dhr6*Cys lines with insertions on the X chromosome. The ratio of Cy^-:Cy^+ progeny from these crosses was not significantly different between sexes, or from 1:1. Overexpressing wild-type DHR6 in the pattern of C22C in crosses to *Dhr6*Cys lines with autosomal insertions (lines 3-9) did not result in a significant difference from the expected 1:1 ratio of Cy^-:Cy^+ flies, except for the females from *Dhr6*Cys lines 3 and 9. In crosses to these lines, there were few or no Cy^+ females produced. As this was seen for just two lines out of nine, and only females are affected, it seems unlikely the affect is due to overexpression of wild-type DHR6 protein, and could be due to the position of insertion of the construct in the genome. Lines overexpressing wild-type DHR6 protein when crossed to C22C did not result in progeny with a significantly different ratio of Cy^-:Cy^+ flies from control lines with no overexpression. Again more Cy^+ than Cy^- flies were seen overall, and Cy^+ progeny had no mutant phenotype. This implies that overexpressing wild-type DHR6 protein throughout all tissues in embryogenesis has no effect on development of flies.

No difference was detected between the progeny from crosses to *Dhr6* Ala and Ser lines, where no Cy^+ progeny were observed when either type of mutant DHR6 protein was overexpressed (table 5.2). As males from the transformed lines were crossed to C22C virgin females, X chromosome insertions could not be inherited by male progeny. The ratio of Cy^- to Cy^+ males in progeny from *Dhr6*Ala line 4 and *Dhr6*Ser lines 2 and 3 is therefore not significantly different from 1:1, or from the results for lines not overexpressing, or overexpressing wild-type, DHR6 protein.

The phenotype for overexpression of Ala or Ser active site mutants of DHR6 protein, in the pattern of the C22C GAL4 line, is lethality.

5.3.2.7 Lethality of overexpression of mutant DHR6 protein may act at the pupal stage:

In order to investigate when overexpression of mutant DHR6 protein was lethal, all transformed lines were crossed to a h.s.GAL4 line and heat shocked for different lengths of time at different stages of development. Flies were kept at 18°C and tipped into fresh vials every other day. Three sets of seven vials for each cross, representing progeny from particular stages, were collected, and one set of each was heat-shocked in a 39°C water bath for 15min, 30min or 45min.

The h.s.GAL4 insertion is on the third chromosome and is balanced by TM3, which carries the dominant marker *stubble* (*Sb*). Any Sb^+ progeny from these crosses should be overexpressing mutant protein. The full results of this experiment can be seen in Appendix I, and the method for statistical analysis on the data is presented in Appendix II. The expected ratio of Sb^- to Sb^+ flies is 1:1 if there is no lethality (figure 5.17). Statistical analysis was performed in order to see whether data for different lines and different variables could be pooled. This was found to be the case for some lines and variables, and pooled results for this experiment are presented in table 5.3. I also tested for significant differences in Sb^-:Sb^+ ratios between male and female progeny, and to see whether the variables of length of time for heat shock, and developmental stage at which progeny were heat shocked, affected the ratio of Sb^-:Sb^+ flies produced. Finally, Sb^-:Sb^+ ratios were compared to the expected 1:1 ratio. Overexpression of mutant DHR6 protein was again seen to affect the viability of flies, although some Sb^+ flies were seen, so lethality was not absolute (table 5.3).

The ratio of Sb^-:Sb^+ progeny was not significantly different between sexes for all crosses except those to *Dhr6*Ala and *Dhr6*Ser transformed lines with X chromosome insertions. In Sb^+ male progeny from crosses to these lines, mutant DHR6 protein is not overexpressed, so significantly more Sb^+ males than females are produced. No significant difference was seen between Sb^+ males and females from crosses to *Dhr6*Cys lines with insertions on the X chromosome; overexpression of wild-type DHR6 protein did not affect viability. Overexpression of mutant DHR6 protein had the same effect in male and female flies.

Control crosses showed heat shocking progeny from crossing the h.s. GAL4 line to a line with no transgene or to lines with wild-type *Dhr6* transgenes under control of the UAS, did not result in a 1:1 ratio of Sb^-:Sb^+ flies. Overall for both types of cross, a significant excess of Sb^+ flies were produced probably due to a reduction in fitness of flies carrying the Sb^- mutation. The results for *Dhr6*Cys lines were significantly different from those for w^{1118}, but there appeared to be no lethality due to overexpression of the wild-type DHR6 protein.

There was lethality when either type of mutant DHR6 protein was overexpressed, and no matter at what stage progeny were heat shocked, even at late pupal stages, an effect on Sb^-:Sb^+ flies was seen. Some Sb^+ flies were produced, probably because the heat shock did not result in uniform levels of expression of mutant protein in all flies, and sometimes the level of mutant protein may not have

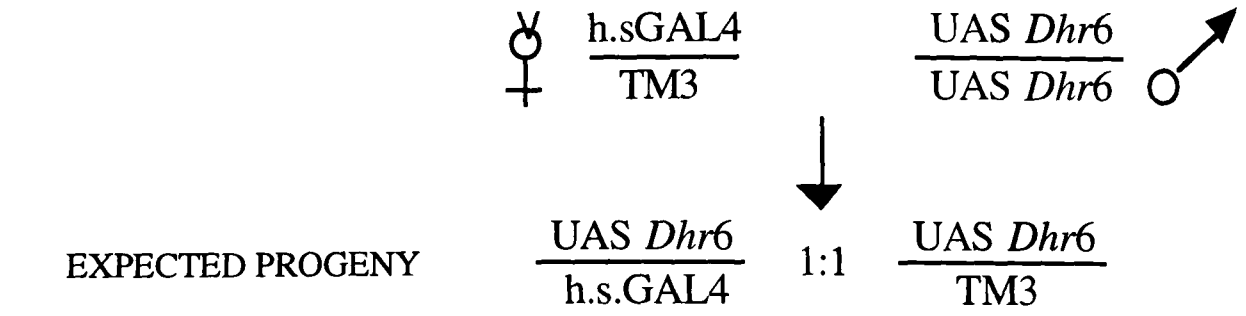


Figure 5.17 h.s.GAL4 crosses to UAS *Dhr6* transformed lines.

	w1118		*Dhr6*Ala line 2		*Dhr6*Cys line 1		*Dhr6*Cys (lines 2, 3, 4, 6, and 9)		*Dhr6*Ala (lines 1, and 3, and 4 females)		*Dhr6*Ser (lines 1, 2 + 3 females, 4, and 5)	
	Sb$^-$	*Sb*$^+$	*Sb*$^-$	*Sb*$^+$	*Sb*$^-$	*Sb*$^+$	*Sb*$^-$	*Sb*$^+$	*Sb*$^-$	*Sb*$^+$	*Sb*$^-$	*Sb*$^+$
VIAL 7 15	59	80	24	60	31	23	134	222	154	56	126	78
30	49	56	61	57	33	23	237	273	196	17	95	36
(E-L1i) 45	53	55	72	12	11	11	245	190	77	16	113	10
VIAL 6 15	52	70	41	58	14	17	183	149	134	26	99	59
30	80	99	68	11	15	11	254	139	165	5	103	17
(L1i-L2i) 45	66	51	36	41	2	2	256	77	65	7	70	12
VIAL5 15	75	111	24	52	14	21	171	223	104	25	124	92
30	70	75	40	39	26	17	219	154	134	5	112	15
(L2i-L3i) 45	77	43	36	18	24	23	218	78	90	2	77	15
VIAL 4 15	123	115	21	38	20	20	203	285	66	33	106	45
30	99	84	21	25	17	19	289	272	114	1	137	6
(L3i-EP) 45	59	59	39	31	13	14	285	226	108	6	107	22
VIAL3 15	69	114	12	27	33	27	154	188	86	10	141	71
30	53	93	21	15	20	26	127	208	105	1	132	9
(EP) 45	26	52	22	38	17	23	122	178	124	2	105	11
VIAL 2 15	79	76	42	51	23	12	154	242	115	16	116	55
30	31	91	22	35	32	29	153	192	90	0	122	14
(EP-LP) 45	11	63	39	39	19	30	133	174	103	3	114	27
VIAL1 15	104	120	41	47	21	27	146	247	112	26	98	35
30	93	126	48	61	29	18	151	192	104	3	104	14
(LP) 45	57	78	50	47	18	16	121	156	70	7	109	12
DHR6 expression	na		No		No		Yes		Yes		Yes	

Table 5.3 Results of h.s GAL4 crosses to *Dhr6* transformed lines.

Data for each sex, and for some *Dhr6* Ala, Cys and Ser lines have been pooled (see Appendix II).

been high enough to kill them. The Sb^{+} flies surviving did not show any obvious mutant phenotype.

The ratio of Sb^{-}:Sb^{+} progeny from crosses to *Dhr6*Ala and *Dhr6*Ser lines differed significantly from that from progeny of crosses to *Dhr6*Cys lines, and from a 1:1 ratio, as very few Sb^{+} flies were produced. Significantly more Sb^{+} flies were present in the progeny from crosses to *Dhr6*Ser lines than from crosses to *Dhr6*Ala lines. This implies that overexpressing a mutant DHR6 protein with Ser at the active site is less severe in its effects on the fly than overexpressing an Ala active site mutant.

Varying the length of time progeny from crosses to *Dhr6*Ala and *Dhr6*Ser lines were heat shocked produced significant differences in the ratio of Sb^{-}:Sb^{+} flies, as significantly more Sb^{+} flies were produced from a 15min heat shock than from heat shocking for 30 or 45 min. This is probably due to more mutant protein being produced when flies are heat shocked for longer periods of time.

Although the stage at which progeny from crosses to *Dhr6*Ala and *Dhr6*Ser lines were heat shocked produced significant differences in the ratio of Sb^{-}:Sb^{+} flies, there appeared to be no pattern to this effect, and this could have been due to the same factors that also made the stage at which progeny were heat shocked a significant variable for control crosses. As significantly less Sb^{+} flies were produced even when progeny were heat shocked at late pupal stages, lethality of overexpression of mutant DHR6 protein appeared to act very late in development.

Uneclosed pupal cases were observed in vials from h.s. GAL4 crosses to *Dhr6*Ala and *Dhr6*Ser lines, from heat shocking progeny at each stage. When these were dissected, fully formed white adults were found inside. It appeared that the flies were unable to eclose. A study on the percentage of flies eclosing after heat shocking for 30min at 39°C at each stage, was performed on crosses to *Dhr6*Ala line 1, *Dhr6*Ala line 3, and w^{1118}. The full results of this experiment and method of statistical analysis performed on the data are presented in Appendix II. The stage at which progeny were heat shocked did not significantly alter the percentage of flies eclosing, so numbers for each stage were pooled (table 5.4). The percentage of eclosion was not significantly different between the progeny from crosses to the *Dhr6*Ala lines, but was significantly different between the progeny of these two crosses, and those from the cross to w^{1118}. This shows a burst of overexpression of mutant DHR6 protein at different stages of development results in death of *Drosophila* at the late pupal stage, but does not exclude the possibility of lethality also acting at other stages. The percentage of eclosion was also scored for all lines in

w^{1118}			*Dhr6*Ala line 1			*Dhr6*Ala line 3		
Pupae	Flies	% Eclosion	Pupae	Flies	% Eclosion	Pupae	Flies	% Eclosion
1003	897	89	647	422	65	521	327	63

Table 5.4 Percentage of flies eclosing, after heat-shocking progeny from w^{1118} and *Dhr6* transformed lines crossed to h.s. GAL4, for 30min at 39°C.

	Sb^-	Sb^+
w^{1118}	88	100
*Dhr6*Ala3	69	47
*Dhr6*Ala4 (X) females	55	39
*Dhr6*Ala4 (X) males	46	68
*Dhr6*Ser1	119	93

Table 5.5 Sb^- and Sb^+ flies produced when progeny from *Dhr6* lines crossed to h.s.GAL4 was not heat shocked.

the C22C experiment, but here eclosion was always greater than 83%, so in these crosses *Drosophila* overexpressing mutant DHR6 protein are dying at an earlier stage.

In order to show the effects on the Sb^-:Sb^+ ratio were due to heat shock induced overexpression of DHR6, progeny from crosses of *Dhr6* transformed lines to the h.s. GAL4 strain were scored without having been heat shocked. Appendix II contains the method used for statistical analysis on the data and full results of this experiment. The results are also presented in table 5.5. Significant differences in the ratio of Sb^-:Sb^+ flies between the two sexes were only seen for progeny from the cross to *Dhr6*Ala line 4 which has an X chromosome insertion. The results from this experiment showed that there was a significant reduction in Sb^+ flies which had the ability to overexpress mutant DHR6 protein under heat shock conditions. In the progeny from the cross to w^{1118}, there were again significantly more Sb^+ than Sb^- flies. Many Sb^+ flies were present in the progeny from crosses to the transformed lines (table 5.5), but the heat shock promoter probably allows basal levels of expression of mutant DHR6 protein, and this significantly reduced the number of Sb^+ flies, though not to levels seen when progeny were heat shocked. One reason why *Drosophila* were seen to die at the late pupal stage, no matter at what developmental stage they were heat shocked, could be due to the basal levels of expression of mutant DHR6 protein.

5.3.2.8 Lethality of overexpression of DHR6 in the pattern of C22C may act at many stages:

As overexpression of mutant DHR6 protein in the pattern of the C22C GAL4 line resulted in death of all *Drosophila*, approximately 50 embryos from crosses of C22C to selected *Dhr6* transformed lines and w^{1118} were lined up and scored for survival through development to identify the stage at which they died. Approximately half of the embryos, from crosses to *Dhr6*Ala and *Dhr6*Ser transformed lines with autosomal insertions, were expected to not be overexpressing mutant DHR6 protein, as the C22C insertion is balanced by SM5. Embryos which carried the SM5 chromosome were expected to survive to become Cy^- flies. Embryos were collected from cages and lined up on coverslips in the same manner as for creating transgenic lines, but without dechorionation. In order to view

development of embryos, they were covered with halocarbon oil which makes the chorion transparent. Any larvae hatching were put into vials of 10-20 per vial. Embryos which did not hatch were scored for their degree of development using the presence of certain morphological structures as evidence for development to a particular stage. The presence of a midgut showed some development had taken place, and embryos which did not have a midgut were grouped as blastoderm stages. Development of the posterior end was scored by the presence of Malphigian tubules, and the presence of mouthparts showed normal development had occured at the anterior end of the embryo. Some embryos appeared to develop into wild-type larvae which were not able to hatch, and these were scored as fully formed larvae. The development of the hatched larvae was scored at the pupal stage, and any adults produced were scored as Cy^- or Cy^+. The results of this experiment are shown in tables 5.6 and 5.7.

Some lethality was seen at each stage tested (table 5.8). The *Dhr6* gene product is probably involved in DNA repair, so when a mutant DHR6 protein is present, the death of *Drosophila* may be due to the accumulation of mutations. This could explain why *Drosophila* die at different stages of development. A lot of death occured at embryonic stages, but the same effects were also seen for crosses to w^{1118}. This was not due to the process of lining up the embryos, as when 50 homozygous w^{1118} embryos were put through this process they all hatched. This implied that the SM5 chromosome was causing embryonic lethality. Heterozygote viability of *Drosophila* carrying the SM5 chromosome is not as good as that of flies carrying the SM1 chromosome 2 balancer (Roberts, 1986). Even though no flies were produced when mutant DHR6 protein was overexpressed as expected, no statistical analysis was performed on the data from this experiment, as many *Drosophila* had died due to carrying the SM5 chromosome.

	EMBRYOS	LARVAE	PUPAE	ADULT FLIES			
				FEMALES		MALES	
				Cy^-	Cy^+	Cy^-	Cy^+
w^{1118}	65	36	23	3	8	5	7
*Dhr6*Cys line 4	56	18	16	0	5	4	3
*Dhr6*Cys line 5	55	41	34	3	12	6	10
*Dhr6*Ala line 1	58	18	8	5	0	3	0
*Dhr6*Ala line 3	53	23	11	5	0	2	0
*Dhr6*Ser line1	48	32	19	8	0	3	0
*Dhr6*Ser line3 (X)	60	29	20	1	0	9	7
*Dhr6*Ser line 4	53	27	15	7	0	8	0

Table 5.6 Survival of progeny from crosses of transformed lines to C22C.

	Blastoderm	Midgut	Malpighian tubules, no mouthparts	Mouthparts, no Malpighian tubules	Mouthparts and Malpighian tubules	Unhatched fully formed larvae
w^{1118}	3	3	1	1	0	21
*Dhr6*Cys line 4	5	6	5	0	4	18
*Dhr6*Cys line 5	2	2	2	0	0	8
*Dhr6*Ala line 1	11	18	6	2	0	3
*Dhr6*Ala line 3	8	15	2	1	1	3
*Dhr6*Ser line 1	3	6	4	0	1	2
*Dhr6*Ser line 3 (X)	7	6	5	3	3	7
*Dhr6*Ser line 4	4	5	15	0	0	2

Table 5.7 Degree of embryonic development seen in the progeny, from C22C crosses to transformed lines, which died as embryos.

	EMBRYOS	LARVAE	PUPAE	SURVIVING TO ADULTS
w^{1118}	45	20	0	35
*Dhr6*Cys line 4	68	4	7	21
*Dhr6*Cys line 5	25	13	6	56
*Dhr6*Ala line 1	69	17	0	14
*Dhr6*Ala line 3	57	23	7	13
*Dhr6*Ser line1	33	27	17	23
*Dhr6*Ser line3 (X)	52	15	5	28
*Dhr6*Ser line 4	49	19	4	28

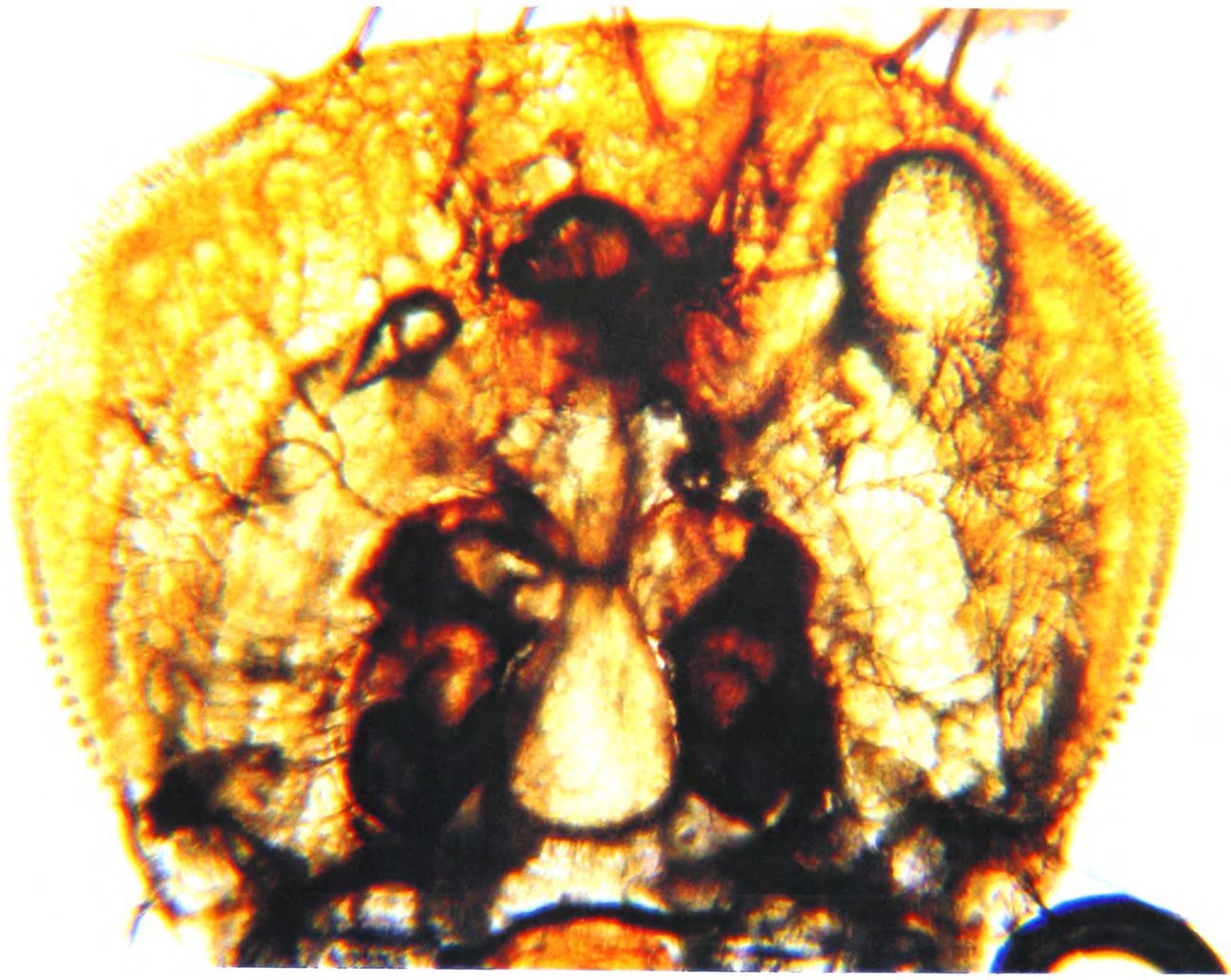
Table 5.8 Percentages of the total progeny from crosses of C22C to transformed lines dying at each stage of development (to the nearest percent).

5.3.2.9 A *ben* transformed line had mosaic eyes due an insertion near heterochromatin:

One *ben* Cys line showed mosaicism for eye colour when the insertion was homozygous (figure 5.18). Heterozygotes showed a uniform pale yellow eye colour implying that the *miniwhite* gene was in a position in the genome where it was unable to be fully expressed. The mosaicism could have been due to position effect, as if the insertion was in a heterochromatic region, in some cells heterochromatinisation could proceed through the insertion, resulting in poor expression of the *w* gene.

In order to test this hypothesis, polytene chromosome squashes were made from salivary glands of larvae from this line, and a biotinylated *ben* cDNA probe was hybridised to them. The*ben* locus has been mapped to 12D1-2 (Thomas and Wyman, 1984; Matuschewski *et al.*, 1996) on the X chromosome. In figure 5.19 ben cDNA can be seen to hybridise to 12D1-2 on the X, and also to 83B-C1 (Lefevre, 1976) on chromosome 3R, in the *ben*Cys line (figure 5.19). This shows the position of the insertion is near to heterochromatin at the chromocentre. A breakpoint in heterochromatin can affect expression of distant euchromatic genes, and when chomosome rearrangements were used to study the range of influence of heterochromatin, it was seen to vary between rearrangements (for review see Weiler and Wakimoto, 1995) . Position effect variegation has been seen to act in an extreme case nearly 2Mb away from the breakpoint. The insertion in the *ben*Cys transformed line is approximately 66 polytene chromosome bands away from heterochromatin, and so is within the range in which position effect variegation can act.

A.



B.

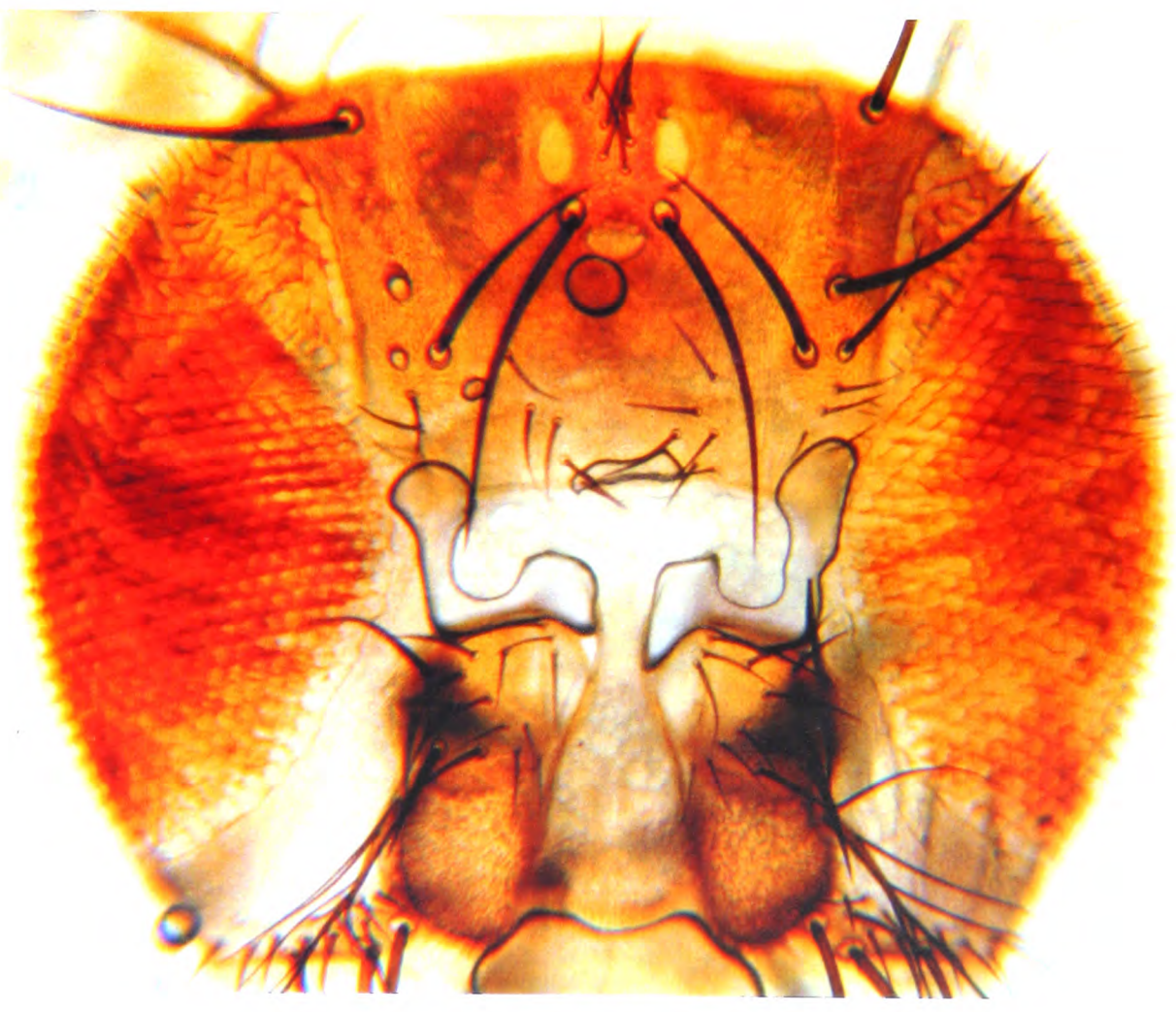
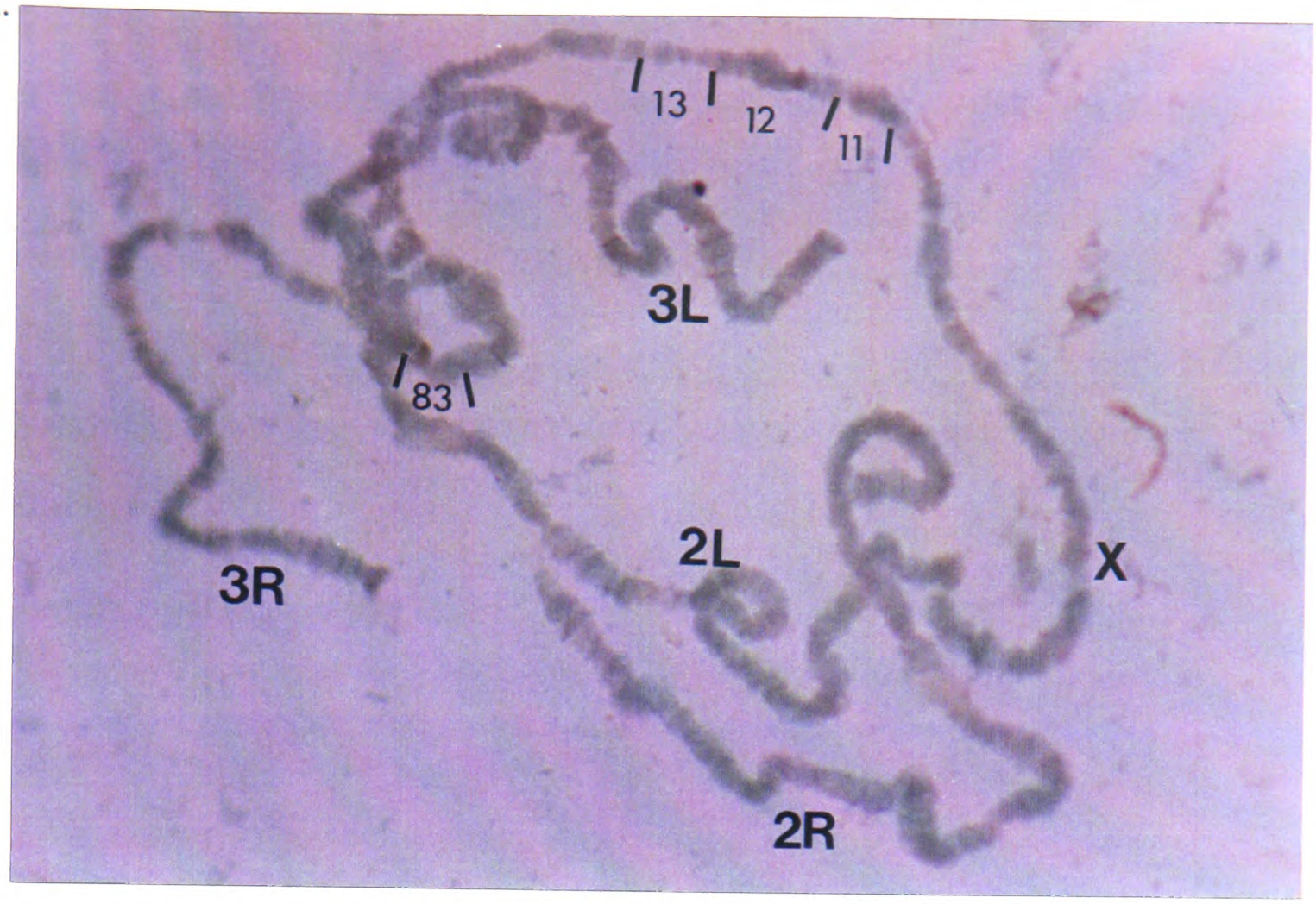


Fig. 5.18 Photographs of *ben*Cys line 2 flies heads mounted in Ringers solution (taken by William Sauer, a Summer student in the lab.).
A. Heterozygous for the insertion; uniform pale yellow eyes.
B. Homozygous for the insertion; mosaic eyes.

A.



B.

Fig. 5.19 Hybridisation of *ben* cDNA to *ben*Cys line2 larval salivary gland polytene chromosomes.
A. Position of w-t *ben* gene, and P insertion are visible.
B. Centromeric regions to show P insertion.
Chromosomes were photographed under bright field optics.

5.3.3 DISCUSSION:

Dhr6 is the RAD6 homologue, so probably has roles in DNA repair mechanisms. If the DHR6 protein is mutant this implies that *Drosophila* may die at a stage when the lack of repair to damaged DNA becomes lethal. Overexpressing mutant DHR6 protein gives a lethal phenotype. It seems likely there is a threshold level of mutant protein expression, above which *Drosophila* will die. In the C22C crosses, there should be a constant high level of overexpression of mutant protein at least throughout embryogenesis. Probably all *Drosophila* die due to this level of overexpression being over the threshold level.

Some flies survive the overexpression of mutant DHR6 in the h.s.GAL4 experiment because there is only a pulse of high level expression, which may not be above the threshold level in all flies. There is also low level expression of mutant DHR6 protein due to the h.s promoter being leaky. Overexpression of mutant DHR6 protein in the h.s.GAL4 experiment resulted in death of *Drosophila* at the late pupal stage, which could also be the stage when the lethality of having a mutant *Dhr6* gene acts. *Dhr6* is expressed in the C.N.S. of developing embryos (chapter 4), as is *ben*, and *ben*$^-$ flies also have an increased likelihood of failing to eclose (Edgecomb *et al.*, 1993): 60% of mutant pupae cannot eclose and often die during emergence (Euk Oh *et al.*, 1994). Perhaps it is defects in the central nervous system, not the lack of DNA repair functions, that leads to flies expressing mutant DHR6 protein being unable to eclose.

These experiments show the DHR6 protein probably performs essential functions, as overexpression of mutant DHR6 protein is lethal. Lethal effects could be due to the overexpressed protein binding to and depleting other factors within each cell, rather than to failure to mediate degradation of particular substrate proteins, but both types of mutant protein had similar effects, and when the wild-type protein is overexpressed it has little or no affect on *Drosophila* development, so this seems unlikely. The active site Cys is necessary for these proposed vital functions, as this is the only part of the protein that was altered and this implies that DHR6 functions are mediated by it acting as an E2 ubiquitin conjugating enzyme. It appears no other residue in DHR6 can act as a site for ubiquitin attachment and exchange, and ubiquitin probably binds irreversibly to Ser when this residue is present at the active site. Overexpressing a mutant DHR6 protein with Ser at the active site was less severe in its effects on *Drosophila* than overexpressing an Ala active site mutant in the crosses to the h.s. GAL4 line. One possible explanation for this observation is that if ubiquitin is bound irreversibly to the Ser active site in most

of the overexpressed mutant DHR6 protein, pools of free ubiquitin in cells may be depleted, which in turn could result in overexpression of ubiquitin from ubiquitin-encoding genes. If there was a ubiquitin deficiency, the Ser mutant would have been expected to have a more severe phenotype than the Ala mutant. If overexpression of ubiquitin does occur, it may be able to slightly suppress the lethality of overexpressing the Ser active site mutant DHR6 protein.

CHAPTER 6

DISCUSSION

6.1 GENERAL DISCUSSION:

The role of E2 enzymes in the life cycle of yeast has been partially determined for most known ubiquitin conjugating enzymes (see 1.7), and shows areas of yeast development and metabolism in which ubiquitination is important. The full significance of protein ubiquitination in a multicellular organism is not known, and work presented in this thesis attempted to address this problem by investigating the ubiquitin conjugating enzymes of *Drosophila melanogaster*.

A new E2, the *Drosophila* homologue of bovine $E2_{25K}$ and possibly also *UBC1* of *S.cerevisiae*, has been discovered. All known E2s were found to express mainly in the developing C.N.S. in embryos, suggesting a role for ubiquitin-dependent proteolysis in development of the nervous system. High levels of mRNA were found for most of these E2s in adult females, probably due to ovarian expression, early embryos and larvae. The ubiquitin system may be particularly important at these stages of development (see below).

At the start of the project, E2s were thought to be the major enzymes of the ubiquitin system for substrate selection, so it was likely any specific developmental role would involve a specific E2. Multiple ubiquitin conjugating enzymes had been discovered in each organism investigated, and yeast E2s were seen to have different functions. E2s are involved in recognising substrates for ubiquitination, often via C-terminal extensions to the UBC domain (Gosink and Vierstra, 1995), but where a class I E2 is necessary for ubiquitination, a ubiquitin protein ligase (E3) may also be involved, and may be the enzyme recognising and binding to the substrate. The C-terminal extensions of class II E2s may be involved in substrate selection by binding a particular E3. E3s are more prevalent than previously thought, and bind tightly to substrates. They are much larger proteins than E2s, so have more domains which could be involved in substrate recognition. Different E3s may bind different substrates, and they are more divergent in sequence than E2s, which all show at least 35% identity across their UBC domains. The "hect" domain E3s become linked to ubiquitin in the same way as E2s (Huibregtse *et al.*, 1995) and show homology across the C-terminal domain, necessary for thiolester formation with ubiquitin, but have divergent N-terminal domains. Ubiquitin is transferred directly to the protein substrate from "hect" ubiquitin protein ligases (Scheffner *et al.*, 1995), and E2 enzymes may only be involved in these ubiquitinations by recognition of, and transfer of ubiquitin to, the E3. Some E2s may function solely as intermediates in the transfer of ubiquitin between E1 and E3 enzymes, or substrate selection may be a

function of complexes between E2 and E3 enzymes. The substrate specificity of some E2s may be due to binding to a subset of E3s, each of which recognises a specific substrate. A large number of E3s would therefore be expected to be present within a eukaryote, and may have more developmental and/or tissue restricted expression than E2 enzymes.

6.2 UBIQUITIN IN *DROSOPHILA* DEVELOPMENT:

To allow specific spatial and temporal distribution of proteins which are key regulators of development, highly selective degradation is required. *Drosophila melanogaster* is a model system for development, and a lot is known about the proteins expressed early in embryogenesis, necessary to set up the embryonic axes. One example is the anterior determinant *bicoid*, whose gene product has a graded distribution in the embryo, dependent on its short half-life (Driever and Nüsslein-Volhard, 1988). Ubiquitin-dependent degradation may control BICOID half-life, and has a proposed role in dorsal-ventral axis formation in the degradation of CACTUS protein to allow the DORSAL transcription factor to enter nuclei on the ventral side of embryos. *Cactus* and *dorsal* are homologues of IκB and NF-κB respectively. Activation of NF-κB, in response to extracellular signals, is controlled by ubiquitin dependent degradation of several proteins (see 1.11.1.2). Other proteins involved in axis formation (review: St Johnston and Nüsslein-Volhard, 1992) probably have short half-lives controlled by the ubiquitin pathway, resulting in spatial restriction to their necessary domains of expression.

Development of distinct tissues and cell types usually requires alterations in the protein composition of the cell, which occurs by protein synthesis and regulated degradation. Proteasomes undergo changes in subunit pattern during *Drosophila* development (Haas and Kloetzel, 1989): the simplest patterns are in early embryos, and subunit pattern becomes increasingly complex with ongoing development. The increase in complexity of proteasomes may be due to an increase in the number of cell types. Ubiquitin-dependent degradation also seems essential for programmed cell death (Schwartz *et al.*, 1990; Haas *et al.*, 1995).

6.2.1 OVARIAN AND EMBRYONIC DEVELOPMENT:

Adult females contain higher levels of E2 mRNA than males (see chapter 3), and this is probably due to levels of expression in the ovaries. The proteins

controlling the axes in ovaries and polarity of developing oocytes are probably controlled in the same way as those for embryonic axes (see above), so E2 expression may be important. The *fat facets* protein is required for embryos to reach cellularization (Fischer-Vize *et al.*, 1992), and is localized at the posterior pole of oocytes, dependent on *oskar*, a gene necessary for posterior determination. The anchoring of a UCH2 family ubiquitin carboxyl terminal hydrolase to the posterior of the developing embryo suggests a role in cleaving ubiquitin from protein substrates that are degraded specifically at the posterior pole.

Expression patterns of *uch-D* suggest a role in oogenesis and early embryogenesis (Zhang *et al.*, 1993), and mRNAs from ubiquitin monomer genes *DUb52* and *DUb80* are also abundant in ovaries (Cabrera *et al.*, 1992; Barrio *et al.*, 1994). There is a high rate of ribosome synthesis during *Drosophila* oogenesis, and the fusion genes encode ribosomal proteins as well as ubiquitin. High levels of UCHD may be necessary to cleave the primary products of *DUb52* and *DUb80*, to allow ribosome assembly, and a high level of free ubiquitin to be present for the start of embryonic protein synthesis.

Levels of E2 mRNAs are high in early embryos. Proteasomes are also thought to be important for early stages of embryogenesis as they can be isolated from transcriptionally inactive embryos (Haass and Kloetzel, 1989) and synthesis of the 20S proteasome is developmentally regulated with the highest concentrations in embryos (Udvardy, 1993). Ubiquitin-dependent degradation in embryos may be involved in cell proliferation and morphogenesis; proteasomes accumulate in gastrulation in cells of the folds involved in morphogenetic movements (Klein *et al.*, 1990).

Ubiquitin dependent proteolysis controls the cell cycle, so alterations in ubiquitination might be responsible for the first ten mitotic divisions in the embryo which are fast and occur in a syncitium. Cyclins are not degraded during the first seven cell cycles (Edgar *et al.*, 1994), but there is a progressive increase in degradation of cyclins at mitosis during cycles 8-13. During interphase 14, programmed degradation of maternal STRING protein leads to inhibitory phosphorylation of CDC2 and cell cycle arrest. *String* gene product is required for mitosis (Edgar and O'Farrall, 1989) and acts as a specific CDC2-activating phosphatase. As STRING is a specifically degraded protein controlling the cell cycle, it seems likely that it is degraded via the ubiquitin pathway. Pulses of zygotic *string* transcription trigger mitoses 14-16 (Edgar *et al.*, 1994), so the protein must have a short, tightly controlled half-life, and the *S.pombe* STRING homologue

CDC25 is degraded via the ubiquitin pathway (Nefsky and Beach, 1996). Control of early embryonic cell divisions may require activity of E2s, which could be one reason for the observation of high levels of mRNA for most known *Drosophila* E2s at this stage. In particular, both transcripts of *Dhr6* are abundant. *Dhr6* is the *Drosophila* homologue of yeast *RAD6*, which may have a cell cycle function, as RAD6 UBC domain can substitute for that of CDC34 (Kolman *et al.*, 1992; Silver *et al.*, 1992).

6.2.2 LARVAL DEVELOPMENT:

UbcD1, *UbcD2*, *ben* and *Dhr6* 2.1kb transcripts are abundant in larval stages of development. The *hyperplastic discs* (*hyd*) gene product has a role at this stage in controlling imaginal disc growth (see 1.8.4). The HYD protein is a "hect" domain E3 which may bind to the *ben* gene product (Amani and Shearn, 1996). Transcription of *ben* may be enhanced in order for the encoded protein to bind to HYD, and mediate ubiquitination of substrate proteins in imaginal discs. The interaction between BEN and HYD may be specific for this function. The *hyd* transcript is specifically expressed in imaginal discs in larvae, and BEN could bind other spatially restricted E3s, in order to perform its other functions in nervous system and eye development (see below). It is not known whether other E2s also bind HYD. They may interact with other E3s and perform different roles.

6.2.3 EYE DEVELOPMENT:

A role for the ubiquitin pathway in eye development is implicated by mutants for *bendless* and *fat facets* genes. In *ben* mutants, the arrangement of photoreceptor rhabdomeres within each ommatidium is altered (Muralidhar and Thomas, 1993). The R7 photoreceptor rhabdomeres are shrunken and outer photoreceptor R1-R6 rhabdomeres are loosely packed and further from the centre of each ommatidium than in a wild type eye. These morphological defects could be due to the fact axons of the photoreceptor neurons initially project normally, but fail to do so later in their pathways: ubiquitination is necessary for growth cone guidance. The eye defects of *faf* mutants are also neuronal in origin: more cells than normal become photoreceptors, resulting in more than the wild type eight photoreceptors in each eye facet (Fischer-Vize *et al.*, 1992). FAF protein is thought to prevent cells becoming neurons by deubiquitinating a protein before it becomes degraded by the proteasome.

The *ben* ubiquitin conjugating enzyme is active in the same cells as FAF, but their functions may not be related.

6.2.4 NERVOUS SYSTEM DEVELOPMENT:

All five *Drosophila* ubiquitin conjugating enzymes investigated were found to have enhanced, if not exclusive, expression of mRNA within the central nervous system (CNS). This implies that ubiquitination is important in the development of the CNS. Possible reasons for increased expression of E2 enzymes in the developing CNS are for roles in axon growth cone guidance, control of cell cycles, and involvement in programmed cell death.

The presence of a wild type *bendless* gene is necessary for the synaptic connection between the giant fibre and tergotrochanter muscle motor neuron (see 1.7.3.3). This is a specific developmental event, and, as *bendless* is expressed throughout the central nervous system, may require the functions of a specific ubiquitin protein ligase recognised by BEN. The *ben*$^{-}$ phenotype corresponds to a presynaptic defect (Euk Oh *et al.*, 1994), probably involving growth cone dysfunction. As mRNA from all the *Drosophila* E2 genes investigated was shown to be concentrated in the CNS, it is possible all have roles in axon guidance of other neurons, and this may be a general function of the ubiquitin system. In *Drosophila*, mutations in the tyrosine kinase genes *abl* and *drl* affect growth cone guidance in the developing CNS (Callahan *et al.*, 1995), and specific receptor tyrosine phosphatases are also necessary for guidance of specific axons (Desai et al., 1996; Krueger et al., 1996). Ligands binding to these receptors could stimulate or repress axon growth by endocytosis of the receptor, and signal transduction via control of tyrosine phosphorylation. The ubiquitin pathway is involved in receptor mediated endocytosis (see 1.12.3), and E2s could function in growth cone guidance to ubiquitinate and mediate endocytosis of ligand-bound receptor tyrosine kinases of specific neurons.

In embryos that have developed past mitosis 16, *string* expression is seen mainly in proliferating neuroblasts of the central and peripheral nervous system (Edgar *et al.*, 1994). If a major role of the ubiquitin system is to control cell cycles, then this could be another explanation for expression of E2 enzymes within the CNS.

Large numbers of cells die by programmed cell death during development of the *Drosophila* central nervous system. Zhou *et al.* (1995) investigated programmed cell death in the central nervous system midline, and found it plays a crucial role in differentiation and is lineage specific. Programmed cell death of *Manduca sexta*

intersegmental muscles involves the ubiquitin system (see 1.12.6) and certain proteins involved in the pathway are induced during this process (Haas *et al.*, 1995). E2s may be upregulated in the CNS in order to function in programmed cell death.

6.2.4.1 SHUTTLE CRAFT could be a substrate for ubiquitination by UBCD4:

When E2$_{25K}$, the bovine homologue of *UbcD4*, was used in a yeast two hybrid screen it was seen to bind to NF-X1 (Pickart, *pers. comm.*). NF-X1 is a gamma interferon-inducible transcription factor implicated in the genetic control of human immunity. It is presumed to limit inflammatory responses by negatively regulating transcription of specific MHC class II molecules. E2$_{25K}$ may ubiquitinate NF-X1, and experiments are currently being performed to determine if NF-X1 is ubiquitinated *in vivo*, and to observe binding of E2$_{25K}$ to NF-X1 *in vitro*.

NF-X1 has a *Drosophila* homologue encoded by the *shuttle craft* (*stc*) gene (Stroumbakis *et al.*, 1996). It is an essential gene; mutant embryos die because they are incapable of coordinating muscle contractions required for hatching. STC protein is normally localized in the nuclei of repeated clusters of cells located in each neuromere along the length of the ventral nerve cord, and to distinct groups of cells within the brain lobes (Stroumbakis *et al.*, 1996). In mutant *stc* embryos, morphological alterations are seen in specific motorneuronal axons. The STC protein may regulate the activity of a subset of genes that play a role in the guidance or spatial maintenance of axon tracts.

UbcD4 mRNA is present at low levels throughout the embryo during development, but is expressed at higher levels in the CNS. If it does bind and ubiquitinate STC, then this is another ubiquitination reaction that controls axon growth of specific neurons within the developing CNS. It is possible ubiquitination acts in axon guidance by activating transcription factors, rather than in receptor mediated endocytosis.

All *Drosophila* E2s show a concentration of mRNA in the CNS, BEN protein affects specific synaptic connections, and UBCD4 may ubiquitinate a protein involved in axon growth. A major role for ubiquitin conjugating enzymes in *Drosophila* development appears to be in growth cone guidance, to allow correct synaptic connections within the developing central nervous system. However, in order to directly determine the role of the E2s in development it is necessary to have mutants.

6.3 MUTAGENESIS OF E2 ENZYMES:

Mutating the active site Cys of DHR6 to Ala or Ser, and overexpression of the mutant protein resulted in a lethal phenotype (see chapter 5). Ubiquitin bound to mutant E2s, with Ser at the active site, was detectable in Western blots, and this concurred with the results of Sullivan and Vierstra (1993) who found ubiquitin bound irreversibly to the Ser residue of an E2 active site mutant. It is difficult to interpret the results of the experiments to determine the stage of development at which *Drosophila* die, due to the lethality of balancer chromosomes used in these experiments.

Overexpressing a dominant negative mutant E2 enzyme does not show the effect of having a mutant gene encoding the enzyme. In order to prove the DHR6 protein performs essential functions it is still necessary to obtain *Dhr6* mutants.

6.4 FUTURE WORK:

Few substrates of ubiquitin conjugating enzymes are known. They could provide a direct means for analysing the role of protein ubiquitination in development. STC may be confirmed as a substrate for ubiquitination by UBCD4, by performing binding studies and *in vitro* ubiquitination assays with purified enzymes. Further possible substrates for ubiquitination by UBCD4 or other known E2s could be identified by yeast two hybrid screens (Fields and Song, 1989) using these enzymes.

If many E3 enzymes are present within a eukaryote, and these are the main enzymes for substrate recognition for the ubiquitin pathway, then it would be interesting to identify further ubiquitin protein ligases in *Drosophila*. A screen could be performed using PCR amplifications with oligonucleotides designed to the conserved C-terminal domain of "hect" proteins. The E3 HYD may bind to the *ben* gene product, and protein binding studies between E2s and HYD/any other E3s discovered could be used to identify which enzymes interact with one another.

It is probable not all the E2s have been isolated from *Drosophila* as yet, and if screens for E3s fail to identify many new enzymes, then E2s may be confirmed as the main enzymes of the ubiquitin pathway for substrate selection. Further E2 enzyme genes could be cloned by designing oligonucleotides to regions other than those used to clone *UbcD4* and performing PCR amplifications.

In order to determine the role of the E2s in development it is necessary to have mutants. Mutating the active site Cys of DHR6 and overexpression of the mutant protein results in lethality. In order to determine whether overexpression of mutant DHR6 is lethal in individual cells, transformed lines could be crossed to GAL4 lines expressing in specific cells of the eye and it could be observed if these cells die. *Dhr6* is expressed in the CNS, and Julie Beattie, an Honours student in the laboratory, is expressing the mutant protein in the CNS by using the GAL4 line 31-1 (Brand and Perrimon, 1993). This will indicate whether expression of *Dhr6* is only important in the CNS. As *ben*$^{-}$ flies, which have defects in the CNS, have an increased likelihood of failing to eclose (Edgecomb, *et al.*, 1993) it would be interesting to dissect the nervous system of flies in which mutant DHR6 protein has been overexpressed, as these flies also seem to die due to failure to eclose. *Dhr6* could be confirmed to be an essential gene by expressing antisense constructs, or creating flies mutant for the gene by P element insertional mutagenesis or by more conventional chemical mutagenesis methods. Creating mutant E2 genes could be used to investigate the hypothesis that they play important roles in nervous system development of *Drosophila*.

Putative target proteins for ubiquitin-dependent degradation in early embryogenesis include STRING and the proteins with short half lives, necessary for establishing embryonic axes. It would be interesting to determine whether these proteins are ubiquitinated *in vivo*, and to view interactions between them and known components of the *Drosophila* ubiquitin pathway.

The levels of *Drosophila* E2 enzyme mRNAs vary across development, indicating that transcription may be developmentally regulated. *In situ* hybridisation to E2 mRNA in embryos revealed high levels were present in the CNS, suggesting that there may be important roles for ubiquitination in nervous system development. Results from other laboratories working on *bendless* also indicate this to be the case. Further investigation into the roles of the ubiquitin system in CNS development could be informative. *UbcD4*, the new E2 discovered, is the homologue of bovine $E2_{25K}$, and may also have a role in CNS development if it is found to ubiquitinate STC. Although it is still necessary for flies with mutant E2 genes to be obtained, in order to directly determine their roles in development, preliminary experiments overexpressing active site mutants of DHR6 show Cys is essential at the active site. Overexpressing active site mutants was lethal, whereas overexpressing wild type DHR6 protein had no obvious effect on viability. *Dhr6* is probably an essential gene.

The results of experiments presented in this thesis concur with the hypothesis that ubiquitin conjugating enzymes, and the ubiquitin system, are involved in *Drosophila* development.

APPENDICES

APPENDIX I: Results of h.s. GAL4 X *Dhr6* lines experiments

w^{1118} X h.s. GAL 4 (combined data from duplicate experiments)

	Sb^-		Sb^+		RATIO
	FEMALE	MALE	FEMALE	MALE	(Sb^-/Sb^+)
VIAL 7 15	25	34	39	41	0.96
30	27	22	28	28	0.88
(E-L1i) 45	34	19	34	21	0.74
VIAL 6 15	27	25	40	30	0.74
30	45	35	58	41	0.81
(L1i-L2i) 45	36	30	30	21	2.25
VIAL5 15	43	32	71	40	0.68
30	36	34	40	35	0.93
(L2i-L3i) 45	43	34	25	18	1.79
VIAL 4 15	75	48	71	44	1.07
30	63	36	48	36	1.18
(L3i-EP) 45	32	27	29	30	1.00
VIAL3 15	29	40	70	44	0.61
30	29	24	67	26	0.57
(EP) 45	15	11	42	10	0.50
VIAL 2 15	47	32	50	26	1.04
30	15	16	51	40	0.34
(EP-LP) 45	6	5	42	21	0.17
VIAL1 15	57	47	60	60	0.87
30	49	44	55	71	0.74
(LP) 45	27	30	46	32	0.73
TOTALS	760	625	996	715	1.15

*Dhr6*Cys line 1 (Y) X h.s. GAL 4

	Sb^-		Sb^+		RATIO (female)	RATIO (male)
	FEMALE	MALE	FEMALE	MALE	(Sb^-/Sb^+)	(Sb^-/Sb^+)
VIAL 7 15	20	11	10	13	2.00	0.85
30	9	14	16	7	0.56	2.00
(E-L1i) 45	5	6	7	4	0.71	1.50
VIAL 6 15	9	5	8	9	1.13	0.56
30	3	12	7	4	0.43	3.00
(L1i-L2i) 45	2	0	2	0	1.00	-
VIAL5 15	9	5	12	9	0.75	0.56
30	13	13	9	8	1.44	1.63
(L2i-L3i) 45	16	8	15	8	1.07	1.00
VIAL 4 15	10	10	11	9	0.82	1.11
30	8	9	9	10	0.90	0.89
(L3i-EP) 45	9	4	12	2	0.75	2.00
VIAL3 15	15	18	13	14	1.15	1.29
30	6	14	13	13	0.46	1.08
(EP) 45	9	8	14	9	0.64	0.89
VIAL 2 15	12	11	5	7	2.40	1.57
30	14	18	15	14	0.93	1.28
(EP-LP) 45	12	7	15	15	0.80	0.47
VIAL1 15	13	8	16	11	0.81	0.73
30	10	19	11	7	0.91	1.12
(LP) 45	8	10	8	8	1.25	1.00
TOTALS	212	210	228	181	0.93	1.16

*Dhr6*Cys line 2 (X) X h.s. GAL 4

		Sb$^-$		Sb$^+$		RATIO (female)	RATIO (male)
		FEMALE	MALE	FEMALE	MALE	(Sb$^-$/Sb$^+$)	(Sb$^-$/Sb$^+$)
VIAL 7	15	3	2	6	5	0.50	0.40
	30	19	13	30	13	0.63	1.00
(E-L1i)	45	18	23	22	16	0.82	1.44
VIAL 6	15	2	3	4	2	0.50	1.00
	30	16	20	20	17	0.80	1.18
(L1i-L2i)	45	9	5	9	8	1.00	0.63
VIAL5	15	7	6	3	6	2.33	1.00
	30	16	12	17	13	0.94	0.93
(L2i-L3i)	45	14	17	12	12	1.17	1.42
VIAL 4	15	8	7	11	13	0.73	0.54
	30	12	10	15	12	0.80	0.83
(L3i-EP)	45	19	21	13	16	1.46	1.31
VIAL3	15	10	8	19	16	0.53	0.50
	30	12	5	16	10	0.75	0.50
(EP)	45	9	5	8	5	0.89	1.00
VIAL 2	15	6	14	28	22	0.21	0.64
	30	16	14	21	22	0.76	0.64
(EP-LP)	45	9	12	23	15	0.39	0.80
VIAL1	15	9	12	26	20	0.35	0.60
	30	12	13	19	16	0.63	0.81
(LP)	45	16	11	19	14	0.84	0.79
TOTALS		242	233	341	273	0.71	0.85

*Dhr6*Cys line 3 X h.s. GAL 4

		Sb$^-$		Sb$^+$		RATIO
		FEMALE	MALE	FEMALE	MALE	(Sb$^-$/Sb$^+$)
VIAL 7	15	26	34	47	36	0.72
	30	40	40	58	33	0.88
(E-L1i)	45	37	24	27	12	1.56
VIAL 6	15	11	11	22	33	0.40
	30	38	16	5	9	3.86
(L1i-L2i)	45	34	31	8	8	4.06
VIAL5	15	32	23	41	28	0.80
	30	28	17	17	11	1.61
(L2i-L3i)	45	30	24	7	5	4.50
VIAL 4	15	19	16	25	18	0.81
	30	36	19	30	26	0.98
(L3i-EP)	45	15	12	32	12	0.61
VIAL3	15	15	13	21	17	0.74
	30	22	9	18	14	0.97
(EP)	45	11	6	38	15	0.32
VIAL 2	15	9	16	17	20	0.68
	30	10	9	23	16	0.49
(EP-LP)	45	20	10	28	16	0.68
VIAL1	15	13	11	26	21	0.51
	30	8	5	8	14	0.59
(LP)	45	12	8	22	10	0.63
TOTALS		466	354	520	374	0.92

*Dhr6*Cys line 4 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	15	10	17	16	0.76
30	13	25	21	17	1.00
(E-L1i) 45	12	17	19	14	0.88
VIAL 6 15	11	8	22	14	0.53
30	25	21	22	16	1.21
(L1i-L2i) 45	25	26	8	2	5.10
VIAL5 15	13	20	28	23	0.84
30	32	32	39	15	1.19
(L2i-L3i) 45	30	20	8	6	3.57
VIAL 4 15	20	18	41	35	0.50
30	30	24	26	20	1.17
(L3i-EP) 45	32	21	24	15	1.36
VIAL3 15	28	27	24	21	1.22
30	24	20	31	22	0.83
(EP) 45	18	23	24	16	1.03
VIAL 2 15	29	15	38	31	0.64
30	13	13	16	15	0.84
(EP-LP) 45	24	22	19	14	1.39
VIAL1 15	24	14	22	31	0.72
30	31	18	30	30	0.82
(LP) 45	21	11	24	18	0.76
TOTALS	470	405	503	391	0.98

*Dhr6*Cys line 5 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	22	7	38	3	0.71
30	12	11	28	16	0.52
(E-L1i) 45	35	28	0	0	-
VIAL 6 15	16	11	43	20	0.43
30	36	16	10	6	3.25
(L1i-L2i) 45	47	28	3	8	6.82
VIAL5 15	49	54	51	48	1.04
30	35	38	39	19	1.26
(L2i-L3i) 45	45	43	46	40	1.02
VIAL 4 15	15	7	25	26	0.43
30	9	8	24	5	0.59
(L3i-EP) 45	19	20	8	6	2.79
VIAL3 15	6	2	16	7	0.35
30	13	0	45	14	0.22
(EP) 45	17	6	13	4	1.35
VIAL 2 15	10	7	15	15	0.57
30	3	2	23	8	0.16
(EP-LP) 45	7	4	15	7	0.50
VIAL1 15	12	16	23	23	0.61
30	14	8	18	9	0.81
(LP) 45	5	6	14	11	0.44
TOTALS	427	322	497	295	0.95

*Dhr6*Cys line 6 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	14	3	28	16	0.39
30	17	10	31	21	0.52
(E-L1i) 45	28	19	37	8	1.04
VIAL 6 15	15	12	25	24	0.60
30	22	28	19	1	2.50
(L1i-L2i) 45	52	18	11	3	5.00
VIAL5 15	18	20	25	16	0.93
30	34	26	8	2	6.00
(L2i-L3i) 45	35	11	2	2	11.50
VIAL 4 15	6	16	18	20	0.58
30	21	14	40	16	0.63
(L3i-EP) 45	20	12	16	12	1.14
VIAL3 15	18	14	23	15	0.84
30	13	9	41	23	0.34
(EP) 45	13	11	27	15	0.57
VIAL 2 15	22	25	26	24	0.94
30	34	29	30	20	1.26
(EP-LP) 45	16	10	21	12	0.79
VIAL1 15	7	9	32	16	0.33
30	15	12	29	14	0.63
(LP) 45	11	11	12	19	0.71
TOTALS	431	319	501	299	0.94

*Dhr6*Cys line 7 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	18	10	17	16	0.85
30	24	17	32	3	1.17
(E-L1i) 45	29	24	0	0	-
VIAL 6 15	26	31	40	34	0.77
30	60	58	39	29	1.73
(L1i-L2i) 45	38	42	29	24	1.51
VIAL5 15	38	22	22	26	1.25
30	31	22	21	5	2.04
(L2i-L3i) 45	50	32	0	0	-
VIAL 4 15	14	20	35	25	0.57
30	13	11	22	15	0.65
(L3i-EP) 45	17	13	17	8	1.20
VIAL3 15	14	14	24	24	0.58
30	14	23	32	21	0.70
(EP) 45	16	9	24	21	0.56
VIAL 2 15	12	9	20	17	0.57
30	13	7	27	17	0.45
(EP-LP) 45	8	9	26	13	0.44
VIAL1 15	17	17	27	19	0.74
30	11	13	27	24	0.47
(LP) 45	11	8	25	10	0.54
TOTALS	474	411	509	351	1.03

*Dhr6*Cys line 8 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	12	12	12	11	1.04
30	14	20	11	16	1.26
(E-L1i) 45	37	26	2	0	31.50
VIAL 6 15	69	78	52	45	1.52
30	21	16	10	6	2.31
(L1i-L2i) 45	45	44	5	6	8.09
VIAL5 15	27	11	9	10	2.00
30	25	15	15	13	1.43
(L2i-L3i) 45	30	27	0	0	-
VIAL 4 15	10	6	14	11	0.64
30	33	18	31	10	1.24
(L3i-EP) 45	24	18	11	7	2.33
VIAL3 15	58	48	69	68	0.77
30	33	53	30	30	1.43
(EP) 45	25	29	25	31	0.96
VIAL 2 15	21	12	32	29	0.54
30	9	4	9	3	1.08
(EP-LP) 45	10	8	4	7	1.64
VIAL1 15	16	13	32	21	0.55
30	30	15	29	15	1.02
(LP) 45	11	3	9	15	0.58
TOTALS	560	476	411	354	1.35

*Dhr6*Cys line 9 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	15	14	29	22	0.57
30	31	22	33	16	1.08
(E-L1i) 45	30	37	17	18	1.91
VIAL 6 15	20	13	46	34	0.41
30	36	32	25	5	2.27
(L1i-L2i) 45	24	32	10	10	2.80
VIAL5 15	15	17	30	23	0.60
30	12	10	14	18	0.69
(L2i-L3i) 45	24	13	15	9	1.54
VIAL 4 15	46	47	47	57	0.89
30	70	53	52	35	1.41
(L3i-EP) 45	67	66	43	43	1.55
VIAL3 15	13	8	17	15	0.66
30	5	8	12	21	0.39
(EP) 45	19	7	20	10	0.87
VIAL 2 15	5	13	24	12	0.50
30	9	6	14	15	0.52
(EP-LP) 45	5	5	15	11	0.38
VIAL1 15	25	22	30	23	0.89
30	19	18	20	12	1.16
(LP) 45	9	11	10	8	1.11
TOTALS	499	454	523	417	1.01

*Dhr6*Cys line10 (X) X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO (female) (Sb⁻/Sb⁺)	RATIO (male) (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE		
VIAL 7 15	10	13	16	12	0.63	0.76
30	37	32	16	12	2.31	2.67
(E-L1i) 45	35	32	0	0	-	-
VIAL 6 15	8	12	10	9	0.80	1.33
30	16	12	8	6	2.00	2.00
(L1i-L2i) 45	25	18	0	0	-	-
VIAL5 15	13	6	16	12	0.81	0.50
30	15	16	8	8	1.88	2.00
(L2i-L3i) 45	14	7	0	0	-	-
VIAL 4 15	12	10	10	23	1.20	0.43
30	10	11	13	19	0.77	0.58
(L3i-EP) 45	13	13	1	2	13.00	6.50
VIAL3 15	20	15	25	31	0.80	0.48
30	12	12	21	28	0.57	0.43
(EP) 45	20	20	4	5	5.00	4.00
VIAL 2 15	13	13	19	15	0.68	0.87
30	15	16	33	28	0.45	0.57
(EP-LP) 45	15	14	19	12	0.79:	1.17
VIAL1 15	25	17	28	29	0.89:	0.59
30	25	27	16	14	1.56:	1.93
(LP) 45	22	22	31	20	0.71	1.10
TOTALS	375	338	294	285	1.28	1.19

*Dhr6*Ala line 1 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE	
VIAL 7 15	45	59	224	15	2.67
30	57	55	11	2	8.62
(E-L1i) 45	22	29	8	7	3.40
VIAL 6 15	27	21	14	2	3.00
30	37	28	1	4	13.00
(L1i-L2i) 45	25	19	4	3	6.29
VIAL5 15	16	18	10	6	2.13
30	26	19	1	4	9.00
(L2i-L3i) 45	27	22	1	1	24.50
VIAL 4 15	12	8	10	0	2.00
30	26	23	0	0	-
(L3i-EP) 45	22	10	4	2	5.33
VIAL3 15	25	13	5	3	4.75
30	33	22	1	0	55.00
(EP) 45	34	22	1	1	28.00
VIAL 2 15	22	17	5	4	4.33
30	16	16	0	0	-
(EP-LP) 45	22	13	1	2	11.67
VIAL1 15	9	7	5	7	1.33
30	20	14	2	0	17.00
(LP) 45	20	15	2	0	17.50
TOTALS	450	543	63	110	5.74

*Dhr6*Ala line 2 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	15	9	28	32	0.40
30	37	24	36	21	1.07
(E-L1i) 45	47	25	7	5	6.00
VIAL 6 15	21	20	32	26	0.71
30	35	33	9	2	6.18
(L1i-L2i) 45	22	14	19	22	0.88
VIAL5 15	14	10	33	19	0.46
30	13	27	24	15	1.03
(L2i-L3i) 45	20	16	14	4	2.00
VIAL 4 15	9	12	15	23	0.55
30	9	12	17	8	0.84
(L3i-EP) 45	21	18	15	16	1.26
VIAL3 15	9	3	21	6	0.44
30	10	11	8	7	1.40
(EP) 45	11	11	25	13	1.73
VIAL 2 15	19	23	15	36	0.82
30	8	14	20	15	0.63
(EP-LP) 45	24	15	30	9	1.00
VIAL1 15	23	18	17	30	0.87
30	33	15	24	37	0.79
(LP) 45	25	25	29	18	1.06
TOTALS	355	425	364	438	0.97

*Dhr6*Ala line 3 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIOS
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	10	8	12	4	1.13
30	22	15	4	0	9.25
(E-L1i) 45	8	8	1	0	16.00
VIAL 6 15	35	21	5	5	5.60
30	25	21	0	0	-
(L1i-L2i) 45	0	5	0	0	-
VIAL5 15	25	29	4	3	7.71
30	30	27	0	0	-
(L2i-L3i) 45	13	12	0	0	-
VIAL 4 15	20	20	8	13	1.90
30	27	18	0	1	45.00
(L3i-EP) 45	36	20	0	0	-
VIAL3 15	23	18	2	0	20.50
30	16	24	0	0	-
(EP) 45	29	28	0	0	-
VIAL 2 15	33	21	5	2	7.71
30	24	19	0	0	-
(EP-LP) 45	27	23	0	0	-
VIAL1 15	40	36	2	2	19.00
30	20	17	1	0	37.00
(LP) 45	18	12	0	0	-
TOTALS	481	369	44	30	11.49

*Dhr6*Ala line 4 (X) X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO (female) (Sb⁻/Sb⁺)	RATIO (male) (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE		
VIAL 7 15	32	16	1	37	32.00	0.43
30	47	23	0	41	-	0.56
(E-L1i) 45	10	8	0	2	-	4.00
VIAL 6 15	30	26	0	45	-	0.58
30	54	36	0	26	-	1.38
(L1i-L2i) 45	16	13	0	3	-	4.33
VIAL5 15	16	23	2	37	8.00	0.62
30	32	27	0	18	-	1.50
(L2i-L3i) 45	16	13	0	3	-	4.33
VIAL 4 15	6	25	2	30	3.00	0.83
30	20	25	0	15	-	1.67
(L3i-EP) 45	20	16	0	14	-	1.14
VIAL3 15	7	7	0	12	-	0.58
30	10	9	0	12	-	0.75
(EP) 45	11	18	0	12	-	1.50
VIAL 2 15	22	19	0	28	-	0.68
30	15	14	0	29	-	0.48
(EP-LP) 45	18	22	0	18	-	1.22
VIAL1 15	20	13	10	41	2.00	0.32
30	27	16	0	26	-	0.62
(LP) 45	5	4	5	5	1.00	0.80
TOTALS	434	373	20	454	21.70	0.82

*Dhr6*Ser line1 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE	
VIAL 7 15	21	9	14	15	1.30
30	9	14	8	6	1.64
(E-L1i) 45	12	15	3	2	5.40
VIAL 6 15	13	14	5	6	2.45
30	17	19	7	2	4.00
(L1i-L2i) 45	8	9	1	3	4.25
VIAL5 15	24	25	24	11	1.40
30	10	13	5	2	3.29
(L2i-L3i) 45	13	11	3	4	3.43
VIAL 4 15	22	22	1	0	44.00
30	21	18	1	0	39.00
(L3i-EP) 45	15	7	2	0	11.00
VIAL3 15	16	23	13	11	1.63
30	16	19	4	2	5.83
(EP) 45	11	11	2	0	11.00
VIAL 2 15	12	15	9	6	1.80
30	11	12	1	2	7.67
(EP-LP) 45	14	9	1	2	7.67
VIAL1 15	11	6	6	3	1.89
30	7	5	2	0	6.00
(LP) 45	5	8	0	1	13.00
TOTALS	283	289	78	112	3.01

*Dhr6*Ser line 2 (X) X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO (female) (Sb⁻/Sb⁺)	RATIO (male) (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE		
VIAL 7 15	42	27	10	28	4.20	0.96
30	27	32	7	25	3.86	1.28
(E-L1i) 45	48	39	4	20	12.00	1.95
VIAL 6 15	21	21	22	21	0.95	1.00
30	20	14	1	6	20.00	2.33
(L1i-L2i) 45	20	17	1	3	20.00	5.67
VIAL5 15	12	5	2	11	6.00	0.45
30	21	12	0	7	-	1.71
(L2i-L3i) 45	12	6	2	2	6.00	3.00
VIAL 4 15	18	16	12	17	1.50	0.94
30	25	26	1	19	25.00	1.37
(L3i-EP) 45	16	12	5	11	3.20:	1.09
VIAL3 15	20	16	5	15	4.00	1.07
30	17	22	0	27	-	0.81
(EP) 45	13	15	1	13	13.00	1.15
VIAL 2 15	21	19	8	15	2.63	1.27
30	-	-	-	-	-	-
(EP-LP) 45	19	20	6	16	3.17	1.25
VIAL1 15	6	6	1	2	6.00	3.00
30	24	1	0	24	-	0.50
(LP) 45	17	15	8	14	2.13	1.07
TOTALS	419	341	96	296	4.36	1.15

*Dhr6*Ser line 3 (X) X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO (female) (Sb⁻/Sb⁺)	RATIO (male) (Sb⁻/Sb⁺)
	FEMALE	MALE	FEMALE	MALE		
VIAL 7 15	17	13	11	14	1.55	0.93
30	13	7	4	6	3.25	1.17
(E-L1i) 45	12	2	0	6	-	0.33
VIAL 6 15	13	23	11	13	1.18	1.77
30	13	13	0	7	-	1.86
(L1i-L2i) 45	10	13	3	7	3.33	1.86
VIAL5 15	11	24	7	16	1.57	1.50
30	11	8	0	5	-	1.60
(L2i-L3i) 45	7	9	0	15	-	0.60
VIAL 4 15	7	10	19	15	0.37	0.67
30	10	9	1	11	10.00	0.82
(L3i-EP) 45	11	16	2	11	5.50	1.45
VIAL3 15	20	13	7	18	2.86	0.72
30	13	17	1	10	13.00	1.70
(EP) 45	22	16	3	17	7.33	0.94
VIAL 2 15	22	19	6	13	3.67	1.46
30	22	14	1	20	22.00	0.70
(EP-LP) 45	15	24	2	16	7.50	1.50
VIAL1 15	18	13	9	21	2.00	0.62
30	10	14	3	25	3.33	0.56
(LP) 45	23	11	0	30	-	0.37
TOTALS	300	288	90	318	3.33	0.91

*Dhr6*Ser line 4 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	7	9	9	9	0.89
30	10	7	3	1	4.25
(E-L1i) 45	4	6	0	1	10.00
VIAL 6 15	12	7	8	2	1.90
30	7	7	3	1	3.50
(L1i-L2i) 45	7	7	0	0	-
VIAL5 15	20	14	17	15	1.06
30	7	19	5	3	3.25
(L2i-L3i) 45	15	6	3	1	5.25
VIAL 4 15	10	11	7	4	1.91
30	14	13	2	0	13.50
(L3i-EP) 45	21	13	9	1	3.30
VIAL3 15	17	22	13	7	1.95
30	15	20	0	0	-
(EP) 45	20	11	2	1	10.33
VIAL 2 15	16	17	11	12	1.43
30	23	14	2	3	7.40
(EP-LP) 45	16	16	8	7	2.13
VIAL1 15	15	13	7	3	2.80
30	7	8	5	2	2.14
(LP) 45	15	15	0	1	30.00
TOTALS	278	254	114	74	2.83

*Dhr6*Ser line 5 X h.s. GAL 4

	Sb⁻		Sb⁺		RATIO
	FEMALE	MALE	FEMALE	MALE	(Sb⁻/Sb⁺)
VIAL 7 15	11	10	5	5	2.10
30	5	10	0	1	15.00
(E-L1i) 45	9	7	0	0	-
VIAL 6 15	7	12	4	1	3.60
30	15	5	2	1	6.67
(L1i-L2i) 45	6	3	3	1	2.25
VIAL5 15	12	6	9	7	1.13
30	13	12	0	0	-
(L2i-L3i) 45	7	6	0	2	6.50
VIAL 4 15	7	9	1	1	8.00
30	20	16	1	0	36.00
(L3i-EP) 45	14	10	2	1	8.00
VIAL3 15	14	9	8	7	1.53
30	16	16	1	1	16.00
(EP) 45	7	13	0	2	10.00
VIAL 2 15	9	4	2	1	4.00
30	18	22	1	4	8.00
(EP-LP) 45	11	14	0	1	25.00
VIAL1 15	13	16	5	1	4.83
30	23	20	1	1	21.50
(LP) 45	12	14	2	0	13.00
TOTALS	249	234	47	38	5.68

APPENDIX II: Statistical analysis on the progeny from crosses of lines containing *Dhr6* transgenes to heat shock and C22C GAL4 lines.

The StatView® Student program (Abacus Concepts Inc.) was used to apply the Goodness of Fit Chi-Square test to the data from the results of crossing *Dhr6* transformed lines to C22C and heat shock GAL4 lines.

The following formula was applied for Chi-Squared analysis:

$$X^2 = \sum \frac{(O-E)^2}{E}$$

O = observed values

E = expected values

Degrees of freedom = the number of classes whose frequency may be assigned arbitrarily.

The same formula was applied where contingency table analysis was necessary. In these cases:

N = number of observations

r = number of rows of the contingency table

c = number of columns of the contingency table

Degrees of freedom = (r-1)(c-1)

and E = CR/N, the expected values

C = column total

R = row total

O = observed value

N = grand total

The StatView® Student program calculates values for X^2 and the associated value for P (the probablity that data fit the hypothesis). If P was greater than 0.05 then data were assumed to be not significantly different.

When many tests were performed between sets of data containing small numbers, in order to see if they could be pooled, at least 15% of the tests had to show a P value less than 0.05 before data were deemed to be significantly different, as in small data sets, numbers can be found to be significantly different by chance.

Contingency table Chi-Square tests, comparing the ratios of Cy^-:Cy^+ flies for a particular variable, were performed on the progeny from C22C crosses to lines containing *Dhr6* transgenes and w^{1118}, to see if data could be pooled. Once data had been pooled, Chi-Square was used to calculate the probability the ratio of Cy^-:Cy^+ flies differed from 1:1.

In the same way, contingency table Chi-Square tests, comparing the ratios of Sb^-:Sb^+ flies for a particular variable, were performed to see if data from the progeny of h.s GAL4 crosses to lines containing *Dhr6* transgenes and w^{1118} could be pooled. The ratios of Sb^-:Sb^+ flies for males and females were compared first. If these were not significantly different, values for males and females were pooled. The significance of the effects of altering the length of the heat shock or the stage of development at which the heat shock was applied were also tested. Once data had been pooled, Chi-Square was used to calculate the probability the ratio of Sb^-:Sb^+ flies differed from 1:1.

When contingency table analysis was performed comparing the ratios of Sb^-:Sb^+ flies between the progeny from h.s GAL4 crosses to *Dhr6*Cys lines, these were found to be significantly different. The *Dhr6*Cys lines 5, 7, 8, and 10 often produced no Sb^+ flies when progeny from crosses to h.s. GAL4 were heat shocked for 45min at early developmental stages. No further statistical analysis was performed on these lines. Statistical analysis to calculate the effects, on the ratio of Sb^-:Sb^+ flies, of overexpressing wild-type DHR6 protein at different stages and for different lengths of time was performed on the progeny from crosses to *Dhr6*Cys lines 2, 3, 4, 6 and 9.

Contigency table Chi-square analysis was performed on the data from the experiment to test the percentage eclosion of flies overexpressing mutant DHR6 protein (table A1). First the percentage eclosion of progeny after heat shocking at each stage was compared for the progeny from each cross, in order to see whether data from all stages could be pooled. Percentage eclosion was then compared between the progeny from different crosses.

The results of the experiment to test the effects of not heat shocking progeny from crosses of h.s. GAL4 to lines containing *Dhr6* transgenes are shown in table A2. Contigency table Chi-square analysis was performed to compare the ratio of Sb^-:Sb^+ flies between male and female progeny, then the ratios of Sb^-:Sb^+ progeny were compared between lines. Finally Chi-Square was used to calculate the probability the ratio of Sb^-:Sb^+ flies differed from 1:1.

Stage	*w*1118			*Dhr6*Ala line 1			*Dhr6*Ala line 3		
	Pupae	Flies	% Eclosion	Pupae	Flies	% Eclosion	Pupae	Flies	% Eclosion
E-L1i	82	76	93	155	125	80	60	41	68
L1i-L2i	161	152	94	86	70	81	53	46	87
L2i-L3i	142	119	84	73	50	68	67	57	85
L3i-EP	171	150	88	92	49	53	98	46	47
EP	110	103	94	110	56	51	100	55	55
EP-LP	105	102	97	61	36	59	67	44	66
LP	232	195	84	70	36	51	76	38	50

Table A1 Percentage of flies eclosing, after heat-shocking progeny from *w*1118 and *Dhr6* Ala lines 1 and 3 crossed to h.s. GAL4, for 30min at 39°C.
E = embryo; L1i = first instar larvae; L2i = second instar larvae; L3i = third instar larvae; EP = early pupae; LP = late pupae.

	Sb-		*Sb*+		RATIO
	FEMALE	MALE	FEMALE	MALE	(Sb^-/Sb^+)
*w*1118	49	39	50	50	0.88
*Dhr6*Ala3	41	28	30	17	1.47
*Dhr6*Ala4 (X)	55	46	39	68	0.94
*Dhr6*Ser1	59	60	55	38	1.28

Table A2 Results from not heat shocking the progeny from crosses of *Dhr6* lines to h.s.GAL4.

REFERENCES

Abrams, J., White, K., Fessler, L., Steller, H., (1993) Programmed cell death during *Drosophila* embryogenesis. *Dev.* **117** pp. 29-43.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J., (1994) In: *Molecular Biology of the Cell.* 3rd Ed. Garland Publishing Inc.

Al-Khodairy, F., Enoch, T., Hagan, I., and Carr, A., (1995) The *Schizosaccharomyces pombe hus5* gene encodes a ubiquitin conjugation enzyme required for normal mitosis. *J. Cell Sci.* **108** pp. 475-86.

Amanai, K., and Shearn, A., (1996) Function of *hyd* as an E3 enzyme homolog in ubiquitin proteasome proteolytic pathway in *Drosophila.* 37th Annual *Drosophila* Research Conference Program and Abstracts Volume. p. 367.

Amsterdam, A., Pitzer, F., and Baumeister, W., (1993) Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis, associated with a role in cell cycle control. *Proc. Natl. Acad. Sci. USA* **90** pp. 99-103.

Arnason, T., and Ellison, M., (1994) Stress resistance in *Saccharomyces cerevisiae* is strongly correlated with assembly of a novel type of multiubiquitin chain. *Mol. Cell Biol.* **14** pp. 7876-83.

Arribas, C., Sampedro, J., and Izquierdo, M., (1986) The ubiquitin genes in *Drosophila melanogaster*: transcription and polymorphism. *Biochim. Biophys. Acta* **868** pp. 119-29.

Ashburner, M., (1989) Preparation of DNA from single flies. In: *Drosophila a Laboratory Manual* Cold Spring Harbor Laboratory Press. pp. 108-9.

Bachmair, A., and Varshavsky, A., (1989) The degradation signal in a short-lived protein. *Cell* **56** pp. 1019-32.

Bachmair, A., Finley, D., and Varshavsky, A., (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234** pp. 179-86.

Bachmair, A., Becker, F., Masterson, R., and Schell, J., (1990) Perturbation of the ubiquitin system causes leaf curling, vascular alterations and necrotic lesions in a higher plant. *EMBO J.* **9** pp. 4543-49.

Baker, R., and Varshavsky, A., (1995) Yeast N-terminal amidase - a new enzyme and component of the N-end rule pathway. *J. Biol. Chem.* **270** pp. 12065-74.

Ball, E., Karlik, C., Beall, C., Saville, D., Sparrow, J., Bullard, B., and Fyrberg, E., (1987) Arthrin, a myofibrillar protein of insect flight muscle, is an actin-ubiquitin conjugate. *Cell* **51** pp. 221-28.

Banerjee, A., Deshaies, R., and Chau, V., (1995) Characterization of a dominant negative mutant of the cell cycle ubiquitin-conjugating enzyme Cdc34. *J. Biol. Chem.* **270** pp. 26209-15.

Barrio, R., del Arco, A., Cabrera, H., and Arribas, C., (1994) Structure and expression of the *Drosophila* ubiquitin-80-amino-acid fusion-protein gene. *Biochem. J.* **302** pp. 237-44.

Bartel, B., Wünning, I., and Varshavsky, A., (1990) The recognition component of the N-end rule pathway. *EMBO J.* **9** pp. 3179-89.

Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C., (1996) Surface hydrophobic residues of multiubiquitin chains essential for protein targeting. *Proc. Natl. Acad. Sci. USA* **93** pp. 861-66.

Belvin, M., Jin, Y., and Anderson, K., (1995) Cactus protein-degradation mediates *Drosophila* dorsal-ventral signalling. *Genes Dev.* **9** pp. 783-93.

Benton, W., and Davis, R., (1977) Screening λgt recombinant clones by hybridization to single plaques *in situ*. *Science* **196** pp. 180-182.

Bergmann, M., (1993) Motor neuron disease/amyotrophic lateral sclerosis - lessons from ubiquitin. *Path. Res. Pract.* **189** pp. 902-12.

Berleth, E., and Pickart, C., (1996) Mechanism of ubiquitin gonjugating enzyme E2-230K: catalysis involving a thiol relay? *Biochem.* **35** pp. 1664-71.

Bernards, R., (1996) Cycling in Switzerland. *Trends Gen.* **12** pp. 158-59.

Bingham, P., Kidwell, M., and Rubin, G., (1982) The molecular basis of P-M hybrid dysgenesis: the role of the P element, a P-strain specific transposon family. *Cell* **29** pp. 995-1004.

Brand, A., and Perrimon, N., (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118** pp. 401-15.

Brand, A., and Perrimon, N., (1994) Raf acts downstream of the EGF receptor to determine the dorsoventral polarity during *Drosophila* oogenesis. *Genes and Dev.* **8** pp. 629-39.

Burch, T., and Haas, A., (1994) Site-directed mutagenesis of ubiquitin. Differential roles for arginine in the interaction with ubiqutin-activating enzyme. *Biochem. USA* **33** pp. 7300-08.

Cabrera, H., Barrio, R., and Arribas, C., (1992) Structure and expression of the *Drosophila* ubiquitin-52-amino-acid fusion-protein gene. *Biochem. J.* **286** pp. 281-88.

Cabrera y Poch, H., Arribas, C., and Izquierdo, M., (1990) Sequence of a *Drosophila* cDNA encoding a ubiquitin gene fusion to a 52-aa ribosomal protein tail. *Nuc. Acid Res.* **18** p. 3994.

Callahan, C., Muralidhar, M., Lundgren, S., Scully, A., and Thomas, J., (1995) Control of neuronal pathway selection by a *Drosophila* receptor protein tyrosine kinase family member. *Nature* **376** pp. 171-74.

Callis, J., (1995) Regulation of protein degradation. *Plant Cell* **7** pp. 845-57.

Callis, J., Carpenter, T., Sun, C., and Vierstra, R., (1995) Structure and evolution of genes encoding polyubiquitin and ubiquitin-like proteins in *Arabidopsis thaliana* ecotype *Columbia*. *Genetics* **139** pp. 921-39.

Cardozo, C., Elenteri, A., and Orlowski, M., (1995) Differences in catalytic activities and subunit pattern of multicatalytic proteinase complexes (proteasomes) isolated from bovine pituitary, lung, and liver - changes in LMP7 and the component necessary for expression of the chymotrypsin-like activity. *J. Biol. Chem.* **270** pp. 22645-51.

Carroll, S., and Scott, M., (1985) Localisation of *fushi tarazu* protein during *Drosophila* embryogenesis. *Cell* **43** pp.47-57.

Chau, V., Tobias, J., Bachmair, A., Marriott, D., Ecker, D., Gonda, D., and Varshavsky, A., (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243** pp.1576-1583.

Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M., (1993) Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MATα2 repressor. *Cell* **74** pp. 357-69.

Chen, Z., Niles, E., and Pickart, C., (1991) Isolation of a cDNA encoding a mammalian multiubiquitinating enzyme ($E2_{25K}$) and overexpression of the functional enzyme in *Escherichia coli*. *J. Biol. Chem.* **266** pp.15698-15704.

Chen, Z., Hugler, J., Palombella, V., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T., (1995) Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.* **9** pp. 1586-97.

Chen, Z., Parent, L., and Maniatis, T., (1996) Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. *Cell* **84** pp. 853-62.

Chomczynski, P., and Sacchi, N., (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162** pp. 156-59.

Ciechanover, A., (1994) The ubiquitin-proteasome proteolytic pathway. *Cell* **79** pp. 13-21.

Ciechanover, A., and Schwartz, A., (1989) How are substrates recognized by the ubiquitin-mediated proteolytic system? *Trends Biochem. Sci.* **14** pp. 483-88.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A., (1982) "Covalent Affinity" purification of ubiquitin-activating enzyme. *J. Biol. Chem.* **257** pp. 2537-42.

Ciechanover, A., Finley, D., and Varshavsky, A., (1984) Ubiquitin dependence of selective protein-degradation demonstrated in the mammalian-cell cycle mutant t.s.85. *Cell* **37** pp. 57-66.

Clark, S., McCarron, M., Love, C., and Chovnick, A., (1986) On the identification of the *rosy* locus DNA in *Drosophila melanogaster* - intragenic recombination mapping of mutations associated with inversions and deletions. *Genetics* **112** pp. 755-67.

Clarke L., and Carbon J., (1976) A colony bank containing synthetic colE1 hybrid plasmids representative of the entire *E.coli* genome. *Cell* **9** pp.91-99.

Cook, J., and Chock, P., (1991a.) Immunocytochemical localization of ubiquitin-activating enzyme in the cell nucleus. *Biochem. Biophys. Res. Comm.* **174** pp. 564-71.

Cook, J., and Chock, P., (1991b.) Association of ubiquitin-activating enzyme with HeLa cell chromosomes during mitosis. *Proc. Natl. Acad. Sci. USA* **88** pp. 11388-92.

Cook, J., and Chock, P., (1992) Isoforms of mammalian ubiquitin-activating enzyme. *J. Biol. Chem.* **267** pp. 24315-21.

Cook, W., Jeffrey, L., Sullivan, M., and Vierstra, R., (1992) Three-dimensional structure of a ubiquitin-conjugating enzyme (E2). *J. Biol. Chem.* **267** pp. 15116-21.

Cook, W., Jeffrey, L., Kasperek, E., and Pickart, C., (1994) Structure of tetraubiquitin shows how multiubiquitin chains can be formed. *J. Mol. Biol.* **236** pp. 601-09.

Cowland, J., Wiborg, O., and Vuust, J., (1988) Human ubiquitin genes - one member of the UBB gene subfamily is a tetrameric non-processed pseudogene. *FEBS Lett.* **231** pp. 187-91.

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., and Baumeister, W., (1989) The multicatalytic proteinase (prosome) is ubiquitinous from eukaryotes to archaebacteria. *FEBS Lett.* **251** pp. 125-31.

Dawson, S., Arnold, J., Mayer, N., Reynolds, S., Billett, M., Gordon, C., Colleaux, L., Kloetzel, P.-M., Tanaka, K., and Mayer, R., (1995) Developmental changes of the 26S-proteasome in abdominal intersegmental muscles of *Manduca sexta* during programmed cell-death. *J. Biol. Chem.* **270** pp. 1850-58.

Dayhoff, M., (1978) Survey of new data and computer methods of analysis. In: *Atlas of protein sequence and structure* ed. Dayhoff, M., Silver Springs, Md.: Natl. Biomed. Res. Found. Vol. **5** (3) pp. 2-8.

DeMartino, G., Moomaw, C., Zagwitko, O., Proske, R., Afendis, S., Swaffield, J., and Slaughter, C., (1994) PA700, an ATP-dependent activator of the 20S proteasome, is an ATPase containing multiple members of a nucleotide-binding protein family. *J. Biol. Chem.* **269** pp. 20878-84.

Desai, C., Gindhart, J., Goldstein, L., and Zinn, K., (1996) Receptor tyrosine phophatases are required for motor axon guidance in the *Drosophila* embryo. *Cell* **84** pp. 599-609.

Deshaies, R., (1995a.) Make it or break it: the role of ubiquitin-dependent proteolysis in cellular regulation. *Trends Cell Biol.* **5** pp. 428-34.

Deshaies, R., (1995b.) The self-destructive personality of a cell cycle in transition. *Curr. Biol.* **7** pp. 781-89.

Deshaies, R., Chau, V., and Kirschner, M., (1995) Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J.* **14** pp. 302-12.

Deveraux, Q., Ustell, V., Pickart, C., and Rechsteiner, M., (1994) A 26S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem.* **269** pp. 7059-61.

Dohmen, R., Madura, K., Bartel, B., and Varshavsky, A., (1991) The N-end rule is mediated by the UBC2 (RAD6) ubiquitin-conjugating enzyme. *Proc. Natl. Acad. Sci. USA* **88** pp. 7351-55.

Dohmen, R., Stappen, R., McGrath, J., Forrová, H., Kolaroc, J., Goffeau, A., and Varshavsky, A., (1995) An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. *J. Biol. Chem.* **270** pp. 18099-109.

Driever, W., and Nüsslein-Volhard, C., (1988) A gradient of bicoid protein in *Drosophila* embryos. *Cell* **54** pp. 83-93.

Driscoll, J., and Goldberg, A., (1990) The proteasome (multicatalytic protease) is a component of the 1500kDa proteolytic complex which degrades ubiquitin-conjugated proteins. *J. Biol. Chem.* **265** pp. 4789-92.

Dubiel, W., Ferrel, K., and Rechsteiner, M., (1995) Subunits of the regulatory complex of the 26S protease. *Mol. Biol. Rep.* **21** pp. 27-34.

Durner, J., and Böger, P., (1995) Ubiquitin in the prokaryote *Anabaena variabilis*. *J. Biol. Chem.* **270** pp. 3720-25.

Ecker, D., Khan, M., Marsh, J., Butt, T., and Crooke, S., (1987) Chemical synthesis and expression of a cassette adapted ubiquitin gene. *J. Biol. Chem.* **262** pp.3524-27.

Edgar, B., and O'Farrell, P., (1989) Genetic-control of cell-division patterns in the *Drosophila* embryo. *Cell* **57** pp. 177-87.

Edgar, B., Sprenger, F., Duronio, R., Leopold, P., and O'Farrell, P., (1994) Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev.* **8** pp. 440-52.

Edgecomb, R., Ghetti, C., and Schneiderman, A., (1993) *Bendless* alters thoracic musculature in *Drosophila*. *J. Neurogen.* **8** pp. 201-19.

Egner, R., and Kuchler, K., (1996) The yeast multidrug transporter PDR5 of the plasma-membrane is ubiquitinated prior to endocytosis and degradation in the vacuole. *FEBS Lett.* **378** pp. 177-81.

Emr, S., Vassarotti, A., Garrett, J., Geller, B., Takeda, M., and Douglas, M., (1986) The amino terminus of the yeast F1-ATPase BETA-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* **102** pp. 523-33.

Engels, W., (1989) P-elements in *Drosophila melanogaster*. In: *Mobile DNA* eds. Berg, D., and Howe, M., Am. Soc. Microbiol. Washington D.C. pp.437-84.

Euk Oh, C., McMahon, R., Benzer, S., and Tanouye, M., (1994) *Bendless*, a *Drosophila* gene affecting neuronal connectivity, encodes a ubiquitin-conjugating enzyme homolog. *J. Neuroscience* **14** (5) pp. 3166-3179.

Eytan, E., Ganoth, D., Armon, T., and Hershko, A., (1989) ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin. *Proc. Natl. Acad. Sci. USA* **86** pp. 7751-55.

Eytan, E., Armon, T., Heller, H., Beck, S., and Hershko, A., (1993) Ubiquitin C-terminal hydrolase activity associated with the 26S protease complex. *J. Biol. Chem.* **268** pp. 4668-74.

Falquet, L., Paquet, N., Frutiger, S., Hughes, G., Hoang-Van, K., and Jaton, J.-C., (1995) cDNA cloning of a human 100kDa deubiquitinating enzyme: the 100kDa human deubiquitinase belongs to the ubiquitin C-terminal hydrolase family 2 (UCH2). *FEBS Lett.* **376** pp. 233-37.

Feanteany, G., Standaert, R., Lane, W., Choi, S., Corey, E., and Schreiber, S., (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268** pp. 726-31.

Fehling, H., Swat, W., Laplace, C., Kühn, R., Rajewsky, K, Müller, U., and von Brehmer, H., (1994) MHC classI expression in mice lacking the proteasome subunit LMP-7. *Science* **265** pp. 1234-37.

Ferber, S., and Ciechanover, A., (1987) Role of arginine-transfer RNA in protein-degradation by the ubiquitin pathway. *Nature* **326** pp. 808-11.

Fields, S., and Song, O., (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340** pp. 245-246.

Finley, D., and Chau, V., (1991) Ubiquitination. *Ann. Rev. Cell. Biol.* **7** pp. 25-69.

Finley, D., Ciechanover, A., and Varshavsky, A., (1984) Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant t.s. 85. *Cell* **37** pp. 43-55.

Finley, D., Özkaynak, E., and Varshavsky, A., (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell* **48** pp. 1035-46.

Finley, D., Bartel, B., and Varshavsky, A., (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* **338** pp. 438-40.

Finley, D., Sadis, S., Monia, B., Boucher, P., Ecker, D., Crooke, S., and Chau, V., (1994) Inhibition of proteolysis and cell cycle progression in a multi-ubiquitination deficient yeast mutant. *Mol. Cell Biol.* **14** pp. 5501-09.

Fischer, J., Giniger, E., Maniatis, T., and Ptashne, M., (1988) GAL4 activates transcription in *Drosophila*. *Nature* **332** pp.853-56.

Fischer-Vize, J., Rubin, G., and Lehmann, R., (1992) The *fat facets* gene is required for *Drosophila* eye and embryo development. *Dev.* **116** pp. 985.

Fitch, W., and Margoliash, E., (1967) Construction of phylogenetic trees. *Science* **155** pp. 279-84.

Foster, L., Loftus, M., and Ross, I., (1990) A novel form of ubiquitin found in the Basidiomycete fungus, *Coprinus congregatus*. *Nuc. Acid Res.* **18** p.6449.

Friedman, H., and Snyder, M., (1994) Mutations in *PRG1*, a yeast proteasome-related gene, cause defects in nuclear division and are suppressed by deletion of a mitotic cyclin gene. *Proc. Natl. Acad. Sci. USA* **91** pp. 2031-35.

Fujiwara, Y., Hatano, K., Hirabayashi, T., and Miyazaki, J.-I., (1994) Ubiquitin C-terminal hydrolase as a putative factor involved in sex differentiation of fish (temperate wrasse, *Halichoeres poecilopterus*). *Differentiation* **56** pp. 13-20.

Genschik, P., Durr, A., and Fleck, J., (1994) Differential expression of several E2-type ubiquitin carrier protein genes at different stages in *Arabidopsis thaliana* and *Nicotiana sylvestris*. *Mol. Gen. Genet.* **244** pp. 548-56.

Ghislain, M., Udvardy, A., and Mann, C., (1993) *S.cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. *Nature* **366** pp. 358-62.

Girod, P.-A., and Vierstra, R., (1993) A major ubiquitin conjugation system in wheat germ extracts involves a 15kDa ubiquitin-conjugating enzyme (E2) homologous to the yeast *UBC4/UBC5* gene products. *J. Biol. Chem.* **268** pp. 955-60.

Girod, P.-A., Carpenter, T., van Nocker, S., Sullivan, M., and Vierstra, R., (1993) Homologs of the essential ubiquitin conjugating enzymes UBC1, 4, and 5 in yeast are encoded by a multigene family in *Arabidopsis thaliana*. *The Plant Journal* **3** (4) pp. 545-552.

Glotzer, M., Murray, A., and Kirschner, M., (1991) Cyclin is degraded by the ubiquitin pathway. *Nature* **349** pp. 132-138.

Goebl, M., Yochem, J., Jentsch, S., McGrath, J., Varshavsky, A., and Byers, B., (1988) The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science* **241** pp.1331-35.

Goebl, M., Goetsch, L., and Byers, B., (1994) The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated *in vivo*. *Mol. Cell Biol.* **14** pp. 3022-39.

Goldberg, A., (1995) Functions of the proteasome: the lysis at the end of the tunnel. *Science* **268** pp. 522-23.

Goldknopf, I., and Busch, H., (1977) Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc. Natl. Acad. Sci. USA* **74** pp. 864-68.

Goldstein, G., Scheid, M., Hammerling, U., Boyse, E., Schlesinger, D., and Niall, H., (1975) Isolation of a polyprotein that has lymphocyte differentiating properties and is probably represented universally in living cells. *Proc. Natl. Ac. Sci. USA* **72** pp. 11-15.

Golic, K., (1994) Local transposition of P Elements in *Drosophila melanogaster* and recombination between duplicated elements using a site-specific recombinase. *Genetics* **137** pp. 551-63.

Gonda, D., Bachmair, A., Wünning, I., Tobias, J., Lane, W., and Varshavsky, A., (1989) Universality and structure of the N-end rule. *J. Biol. Chem.* **264** pp. 16700-12.

Gonen, H., Stancovski, I., Sukedy, D., Hadari, T., Bercovich, B., Bengal, E., Mesilati, S., Abu-Hatoum, O., Schwartz, A., and Ciechanover, A., (1996) Isolation, characterization, and partial purification of a novel ubiquitin-protein ligase, E3. *J. Biol. Chem.* **271** pp. 302-10.

Gordon, C., McGurk, G., Dillon, P., Rosen, C., and Hastie, N., (1993) Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. *Nature* **366** pp. 355-57.

Gottesman, S., and Maurizi, M., (1992) Regulation by proteolysis - energy dependent proteases and their targets. *Microbiol. Rev.* **56** pp. 592-621.

Gosink, M., and Vierstra, R., (1995) Redirecting the specificity of ubiquitination by modifying ubiquitin-conjugating enzymes. *Proc. Natl. Acad. Sci. USA* **92** pp. 9117-21.

Gregori, L., Poosch, M., Cousins, G., and Chau, V., (1990) A uniform isopeptide-linked multiubiquitin chain is sufficient to target substrate for degradation in ubiquitin-mediated proteolysis. *J. Biol. Chem.* **265** pp. 8354-57.

Grenfell, S., Trausch-Azar, J., Handley-Gearhart, P., Ciechanover, A., and Schwartz, A., (1994) Nuclear localization of the ubiquitin-activating enzyme, E1, is cell cycle dependent. *Biochem. J.* **300** pp. 701-08.

Gropper, R., Brandt, R., Elias, S., Bearer, C., Mayer, A., Schwartz, A., and Ciechanover, A., (1991) The ubiquitin-activating enzyme, E1, is required for stress-induced lysosomal degradation of cellular proteins. *J. Biol. Chem.* **266** pp. 3602-10.

Gwozd, C., Arnason, T., Cook, W., Chau, V., and Ellison, W., (1995) The yeast UBC4 ubiquitin conjugating enzyme monoubiquitinates itself *in vivo*: evidence for an E2-E2 homointeraction. *Biochem*. **34** pp. 6296-302.

Haas, A., and Rose, I., (1982) The mechanisms of ubiquitin activating enzyme. *J. Biol. Chem.* **257** pp. 10329-37.

Haas, A., Warms, H., Hershko, A., and Rose, I., (1982) Ubiquitin-acitvating enzyme - mechanism and role in protein-ubiquitin conjugation. *J. Biol. Chem.* **257** pp. 2543-48.

Haas, A., Bright Reback, P., and Chau, V., (1991) Ubiquitin conjugation by the yeast *RAD6* and *CDC34* gene products. *J. Biol. Chem.* **266** pp. 5104-12.

Haas, A., Baboshina, O., Williams, B., and Schwartz, L., (1995) Coordinated induction of the ubiquitin conjugation pathway accompanies the developmentally programmed death of insect skeletal muscle. *J. Biol. Chem.* **270** pp. 9407-12.

Haass, C., and Kloetzel, P.-M., (1989) The *Drosophila* proteasome undergoes changes in its subunit pattern during development. *Exp. Cell. Res.* **180** pp. 243-52.

Haass, C., Pesold-Hurt, B., Beyreuther, K., and Kloetzel, P.-M., (1990) The *Drosophila Pros-29* gene is a new member of the *PROS*-gene family. *Nucl. Acids Res.* **18** p. 4018.

Hadari, T., Warms, J., Rose, I., and Hershko, A., (1992) A ubiquitin C-terminal isopeptidase that acts on polyubiquitin chains. *J. Biol. Chem.* **267** pp. 716-27.

Haldeman, M., Finley, D., and Pickart, C., (1995) Dynamics of ubiquitin conjugation during erythroid differentiation *in vitro*. *J. Biol. Chem.* **16** pp. 9507-16.

Hanahan, D., (1985) In: *DNA cloning: a practical approach* vol. I. ed. Glover, D., IRL Press, Oxford pp. 49-78.

Handley, P., Mueckler, M., Siegel, N., Ciechanover, A., and Schwartz, A., (1991) Molecular cloning, sequence, and tissue distribution of the human ubiquitin-activating enzyme E1. *Proc. Natl. Acad. Sci. USA* **88** pp. 258-262.

Handley-Gearhart, P., Stephen, A., Trausch-Azar, J., Ciechanover, A., and Schwartz, A., (1994) Human ubiquitin-activating enzyme, E1: indication of potential nuclear and cytoplasmic subpopulations using epitope-tagged cDNA constucts. *J. Biol. Chem.* **269** pp. 33171-78.

Hatfield, P., and Vierstra, R., (1989) Ubiquitin-dependent proteolytic pathway in wheat-germ - isolation of multiple forms of ubiquitin-activating enzyme, E1. *Biochem*. **28** pp. 735-42.

Hatfield, P., and Vierstra, R., (1992) Multiple forms of ubiquitin-activating enzyme E1 from wheat. Identification of an essential cysteine by *in vitro* mutagenesis. *J. Biol. Chem.* **267** pp. 14799-803.

Hatfield, P., Callis, J., and Vierstra. R., (1990) Cloning of ubiquitin activating enzyme from wheat, and expression of a functional protein in *Escherichia coli*. *J. Biol. Chem.* **265** pp. 15813-17.

Hazelrigg, T., Levis, R., and Rubin, G., (1984) Transformation of *white* locus DNA in *Drosophila*: dosage compensation, *zeste* interaction, and position effects. *Cell* **36** pp. 469-81.

Heinemeyer, W., Gruhler, A., Möhde, V., Mahe, Y., and Wolf, D., (1993) *PRE2*, highly homologous to the human major histocompatability complex-linked *RING10* gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degradation of ubiquitinated proteins. *J. Biol. Chem.* **268** pp. 5115-20.

Heller, H., and Hershko, A., (1989) Binding-sites of ubiquitin-protein ligase - binding of ubiquitin-protein conjugates, and of ubiquitin-carrier protein. *J. Biol. Chem.* **265** pp. 6532-35.

Hershko, A., (1991) The ubiquitin pathway for protein degradation. *Trends Biochem. Sci.* **16** pp. 265-68.

Hersko, A., and Heller, H., (1985) Occurence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem. Biophys. Res. Comm.* **128** pp. 1079-86.

Hershko, A., and Ciechanover, A., (1992) The ubiquitin system for protein degradation . *Ann. Rev. Biochem.* **61** pp. 761-807.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A., (1983) Components of ubiquitin-protein ligase system - resolution, affinity purification and role in protein breakdown. *J. Biol. Chem.* 261 pp. 8206-14.

Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R., and Cohen, L., (1991) Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. *J. Biol. Chem.* **266** pp. 376-79.

Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L., Luca, F., Ruderman, J., and Eytan, E., (1994) Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. *J. Biol. Chem.* **269** pp. 4940-46.

Hicke, L., and Riezman, H., (1996) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **84** pp. 277-87.

Hill, C., Johnston, N., and Cohen, R., (1993) Crystal structure of a ubiquitin-dependent degradation substrate: a 3-disulfide form of lysozyme. *Proc. Natl. Acad. Sci. USA* **90** pp. 4136-40.

Hilt, W., and Wolf, D., (1995) Proteasomes of the yeast *S.cerevisiae*: genes, structure and functions. *Mol. Biol. Rep.* **21** pp. 3-10.

Hingamp, P., Arnold, J., Mayer, J., and Dixon, L., (1992) A ubiquitin conjugating enzyme encoded by African Swine Fever Virus. *EMBO J.* **11** pp. 361-66.

Hochstrasser, M., (1992) Ubiquitin and intracellular protein degradation. *Current Opinion Cell Biol.* **4** pp.1024-31.

Hochstrasser, M., (1994) The DOA pathway: ubiquitin-dependent degradation of the yeast MATα2 repressor. EMBO-RAPPAPORT Workshop.

Hochstrasser, M., (1996) Protein degradation or regulation: Ub the judge. *Cell* **84** pp. 813-15.

Hochstrasser, M., and Varshavsky, A., (1990) *In vivo* degradation of a transcriptional regulator: the yeast α2 repressor. *Cell* **61** pp. 697-708.

Hochstrasser, M., Ellison, M., Chau, V., and Varshavsky, A., (1991) The short-lived MATα2 transcriptional regulator is ubiquitinated *in vivo*. *Proc. Natl. Acad. Sci. USA* **88** pp. 4606-10.

Hodgins, R., Ellison, K., and Ellison, M., (1992) Expression of a ubiquitin derivative that conjugates to protein irreversibly produces phenotypes consistent with a ubiquitin deficiency. *J. Biol. Chem.* **267** pp.8807-12.

Holloway, S., Glotzer, M., King, R., and Murray, A., (1993) Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* **73** pp. 1393-402.

Holmes, D., and Quigley, M., (1981) A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114** pp. 193-97.

Hoshizaki, D., (1994) *Krüppel* expression during postembryonic development of *Drosophila*. *Dev. Biol.* **163** pp.133-140.

Huang, Y., Baker, R., and Fischer-Vize, J., (1995) Control of cell fate by a deubiquitinating enzyme encoded by the *fat facets* gene. *Science* **270** pp. 1828-31.

Huibregtse, J., Scheffner, M., and Howley, P., (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus type-16 or type-18. *EMBO J.* **10** pp. 4129-35.

Huibregtse, J., Scheffner, M., Beaudenon, S., and Howley, P., (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin protein ligase. *Proc. Natl. Acad. Sci. USA* **92** pp. 2563-67.

Hurtley, S., (1996) Lysosomal degradation of ubiquitin-tagged receptors. *Science* **271** p. 617.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K., (1995) Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81** pp. 269-71.

Izquierdo, M., (1994) Ubiquitin genes and ubiquitin protein location in polytene chromosomes of *Drosophila*. *Chromosoma* **103** pp. 193-97.

Izquierdo, M., Arribas, C., Galceran, J., Burke, J., and Cabrera, V., (1984) Characterization of a *Drosophila* repeat mapping at the early-ecdysone puff 63F and present in many eucaryotic genomes. *Biochim. Biophys. Acta* **783** pp. 114-21.

Jensen, J., Bates, P., Yang, M., Vierstra, R., and Weissman, A., (1995) Identification of a family of closely related human ubiquitin conjugating enzymes. *J. Biol. Chem.* **270** pp. 30408-14.

Jensen, T., Loo, M., Pind, S., Williams, D., Goldberg, A., and Riordan, J., (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83** pp. 129-35.

Jentsch, S., (1992) The ubiquitin-conjugation system. *Ann. Rev. Genet.* **26** pp. 177-205.

Jentsch, S., and Schlenker, S., (1995) Selective protein degradation: a journey's end within the proteasome. *Cell* **82** pp. 881-84.

Jentsch, S., McGrath, J., and Varshavsky, A., (1987) The yeast DNA repair gene *RAD6* encodes a ubiquitin-conjugating enzyme. *Nature* **329** pp. 131-34.

Jentsch, S., Seufert W., Sommer, T., and Reins, H.-A., (1990) Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells. *TIBS* **15** pp.195-98.

Jentsch, S., Seufert, W., and Hauser, H.-P., (1991) Genetic analysis of the ubiquitin system. *Biochim. Biophys. Acta* **1089** pp. 127-39.

Johnson, E., Gonda, D., and Varshavsky, A., (1990) Cis-trans recognition and subunit-specific degradation of short-lived proteins. *Nature* **346** pp. 287-91.

Johnson, E., Bartel, B., Seufert, W., and Varshavsky, A., (1992) Ubiquitin as a degradation signal. *EMBO J.* **11** (2) pp.497-505.

Johnson, E., Ma, P., Ota, I., and Varshavsky, A., (1995) A proteolytic pathway that recognises ubiquitin as a degradation signal. *J. Biol. Chem.* **270** pp. 17442-56.

Jones, D., Stringham, E., Graham, R., and Candido, E., (1995) A portable regulatory element directs specific expression of the *Caenorhabditis elegans* ubiquitin gene *UBQ-2* in the somatic gonad. *Dev. Biol.* **171** pp. 60-72.

Jones, M., Haire, M., Kloetzel, P.--M., Mykles, D., and Schwartz, L., (1995) Changes in the structure and function of the multicatalytic proteinase (proteasome) during programmed cell-death in the intersegmental muscles of the hawkmoth, *Manduca sexta. Dev. Biol.* **169** pp. 436-47.

Jungmann, J., Reins, H.-A., Schobert, C., and Jentsch, S., (1993) Resistance to cadmium mediated by ubiquitin-dependent proteolysis. *Nature* **361** pp. 369-71.

Kaiser, K., and Goodwin, S., (1990) "Site-selected" transposon mutagenesis of *Drosophila. Proc. Natl. Ac. Sci. USA* **87** pp. 1686-1690.

Kaiser, P., Mansour, H., Greeten, T., Auer, B., Schweiger, M., and Schneider, R., (1994a.) The human ubiquitin-conjugating enzyme UbcH1 is involved in the repair of U.V. damaged, alkylated and cross-linked DNA. *FEBS Lett.* **350** pp. 1-4.

Kaiser, P., Seufert, W., Hofferer, L., Kofler, B., Sachsenmaier, C., Herzog, H., Jentsch, S., Schweiger, M., and Schneider, R., (1994b) A human ubiquitin-conjugating enzyme homologous to yeast *UBC8. J. Biol. Chem.* **269** pp. 8797-802.

Kaiser, P., Mandl, S., Schweiger, M., and Schneider, R., (1995) Characterization of functionally independent domains in the human ubiquitin conjugating enzyme UbcH2. *FEBS Lett.* **377** pp. 193-96.

Kalfayan, L., and Wensink, P., (1982) Developmental regulation of *Drosophila* α-tubulin genes. *Cell* **29** pp.91-98.

Kang, X., Yadao, F., Gietz, R., Kunz, B., (1992) Elimination of the yeast RAD6 ubiquitin conjugase enhances base-pair transitions and G.C to T.A transversions as well as transposition of the Ty element: implications for the control of spontaneous mutation. *Genetics* **130** pp. 285-94.

Kaplon, T., and Jacquet, M., (1995) The cellular content of CDC25p, the RAS exchange factor in *Saccharomyces cerevisiae*, is regulated by destabilization through a cyclin destruction box. *J. Biol. Chem.* **270** pp. 20742-47.

Karess, R., and Rubin, G., (1984) Analysis of P transposable element functions in *Drosophila. Cell* **38** pp. 135-46.

Kawahara, H., and Yokosaura, H., (1992) Cell cycle-dependent change of proteasome distribution during embryonic development of the Ascidian *Halocynthia roretzi*. *Dev. Biol.* **151** pp. 27-33.

Kay, G., Ashworth, A., Penny, G., Dunlop, M., Swift, S., Brockdorff, N., and Rastan, S., (1991) A candidate spematogenesis gene on the mouse Y chromosome is homologous to ubiquitin-activating enzyme E1. *Nature* **354** pp. 486-89.

Kazahito, A., and Shearn, A., (1996) Function of *hyd* as an E3 enzyme homolog in the ubiquitin proteasome proteolytic pathway in *Drosophila*. Abstract 37th Ann. *Drosophila* Res. Conference.

Kidwell, M., (1986) P-M mutagenesis. In: *Drosophila a Practical Approach* ed. Roberts, D., IRL Oxford pp.59-81.

King, R., Peters, J., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M., (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin-B. *Cell* **81** pp. 279-88.

Klein, U., Gernold, M., and Kloetzel, P.-M., (1990) Cell specific accumulation of *Drosophila* proteasomes (MCP) during early development. *J. Cell. Biol.* **111** pp. 2275-82.

Kölling, R., and Hollenberg, C., (1994) The ABC-transporter Ste6 accummulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. *EMBO J.* **13** pp. 3261-71.

Koken, M., Reynolds, P., Bootsma, D., Hoeijmakers, J., Prakash, S. and Prakash, L., (1991 a.) *Dhr6*, a *Drosophila* homolog of the yeast DNA-repair gene RAD6. *Proc. Natl. Ac. Sci. USA* **88** pp.3832-36.

Koken M., Reynolds, P., Jaspers-Dekker, I., Prakash, L., Prakash, S., Bootsma, D. and Hoeijmakers, J., (1991 b.) Structural and functional conservation of 2 human homologs of the yeast DNA repair gene *RAD6*. *Proc. Natl. Ac. Sci. USA* **88** pp.8865-69.

Koken, M., Smit, E., Jaspers-Dekker, I., Oostra, B., Hagemeijer, A., Bootsma, D., and Hoeijmakers, J., (1992) Localization of 2 human homologs, *HHR6A* and *HHR6B*, of the yeast DNA repair gene *RAD6* to chromosomes Xq24-q25 and 5q23-q31. *Genomics* **12** pp. 447-53.

Koken, M., Hoogerbrugge, J., Jaspers-Dekker, I., de Wit, J., Willemsen, R., Roest, H., Grootegoed, J., and Hoeijmakers, J., (1996) Expression of the ubiquitin-conjugating DNA repair enzymes HHR6A and B suggests a role in spermatogenesis and chromatin modification. *Dev. Biol.* **173** pp. 119-132.

Kolman, C., Toth, J., and Gonda, D., (1992) Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. *EMBO J.* **11** (8) pp.3081-90.

Kong, S., and Chock, P., (1992) Protein ubiquitination is regulated by phosphorylation. An *in vitro* study. *J. Biol. Chem.* **267** pp. 14189-92.

Kong, S., and Chock, P., (1994) Purification and characterization of a ubiquitin carrier protein kinase from HeLa cells. *Proc. Natl. Acad. Sci. USA* **91** pp. 11601-05.

Kornitzer, D., Raboy, B., Kulka, R., and Fink, G., (1994) Regulated degradation of GCN4 is dependent upon the ubiquitin-conjugating enzymes CDC34 (UBC3) and RAD6 (UBC2). *EMBO J.* **13** pp. 6021-30.

Krueger, N., Van Vactor, D., Wan, H., Gelbart, W., Goodman, C., and Saito, H., (1996) The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. *Cell* **84** pp. 611-22.

Kuhner, M., and Felsenstein, J., (1994) A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. *Mol. Biol. Evol.* **11** pp. 459-68.

Lamb, J., Michand, W., Sikorsky, R., and Hieter, P., (1994) Cdc16p, Cdc23p, and Cdc27p form a complex essential for mitosis. *EMBO J.* **13** pp. 4321-28.

Lahavbaratz, S., Sudakin, V., Ruderman, J., Hershko, A., (1995) Reversible phosphorylation controls the activity of a cyclosome-associated cyclin-ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* **92** pp. 9303-07.

Lee, H., Simon, J., and Lis, J., (1988) Structure and expression of ubiquitin genes of *Drosophila melanogaster. Mol. Cell Biol.* **8** pp. 4727-35.

Lenk, S., Dunn, W., Trausch, J., Ciechanover, A., and Schwartz, A., (1992) Ubiquitin-activating enzyme, E1, is associated with maturation of autophagic vacuoles. *J. Cell. Biol.* **118** pp.301-08.

Lefevre, G., (1976) A photographic representation and interpretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands. In: *The genetics and biology of Drosophila 1a* ed. Ashburner, M., and Novitski, E., Academic Press. pp.32-68.

Leyser, H., Lincoln, C., Timpte, C., Lammer, D., Turner, J., and Estelle, M., (1993) *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364** pp. 161-64.

Lindsley, D., and Grell, E., (1968) Genetic variations of *D.melanogaster.* Carnegie Institute of Washington, Publication No. 627.

Lindsley, D., and Zimm, G., (1992) The Genome of *Drosophila melanogaster.* Academic Press.

Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R., (1995) Crystal structure of the 20S proteasome from the Archaeon *T.acidophilaum* at 3.4 Â resolution. *Science* **268** pp. 533-39.

Madura, K., Dohmen, R., and Varshavsky, A., (1993) N-recognin/Ubc2 interactions in the N-end rule pathway. *J. Biol. Chem.* **268** pp. 12046-54.

Mahowald, A., and Kambysellis, M., (1980) Oogenesis. In: *The genetics and biology of Drosophila* ed. Ashburner, M., and Wright, T., Academic Press. Vol. 2d, pp.141-224.

Manetto, V., Perry, G., Tabation, M., Mulvihill, P., Fried, V., Smith, H., Gambetti, P., and Autilio-Gambetti, L., (1988) Ubiquitin is associated with abnormal cytoplasmic filaments characteristic of neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* **85** pp. 4501-05.

Maniatis, T., Fritsch, E., and Sambrook, J., (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratories, Cold Spring Harbour, New York.

Mansfield, E., Hersperger, E., Biggs, J., and Shearn, A., (1994) Genetic and molecular analysis of *hyperplastic discs*, a gene whose product is required for regulation of cell proliferation in *Drosophila melanogaster* imaginal discs and germ cells. *Dev. Biol.* **165** pp. 507-26.

Matuschewski, K., Hauser, H.-P., Treier, M., and Jentsch, S., (1996) Identification of a novel family of ubiquitin-conjugating enzymes with distinct amino-terminal extensions. J. Biol. Chem. **271** pp. 2789-94.

Maurizi, M., (1994) Ubiquitin and intracellular protein degradation. EMBO-RAPPAPORT Workshop.

Mayer, A., and Wilkinson, K., (1989) Detection, resolution, and nomenclature of multiple ubiquitin carboxyl-terminal esterases from bovine calf thymus. *Biochem*. **28** pp. 166-72.

Mayer, A., Siegel, N., Schwartz, A., and Ciechanover, A., (1989) Degradation of proteins with acetylated amino termini by the ubiquitin system. *Science* **244** pp. 1480-83.

McGrath, J., Jentsch, S., and Varshavsky, A., (1991) *UBA1*: an essential yeast gene encoding ubiquitin-activating enzyme. *EMBO J.* **10** pp. 227-36.

Michalek, M., Grant, E., Gramm, C., Goldberg, A., and Rock, K., (1993) A role for the ubiquitin-dependent proteolytic pathway in MHC classI-restricted antigen presentation. *Nature* **363** pp. 552-54.

Miller, H., Henzel, W., Ridgway, J., Kuang, W.-J., Chrisholm, V., and Lin, C.-C., (1989) Cloning and expression of a yeast ubiquitin-protein cleaving activity in *Escherichia coli. Biotech.* **7** pp. 698-704.

Mirabito, P., and Morris, N., (1993) BIMA, a TPR-containing protein required for mitosis, localizes to the spindle pole body in *Aspergillus nidulans. J. Cell. Biol.* **120** pp. 959-68.

Mitchell, M., Woods, D., Tucker, P., Opp, J., and Bishop, C., (1991) Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1. *Nature* **354** pp. 483-86.

Monaco, J., and McDevitt, H., (1986) The LMP antigens: a stable MHC-controlled multisubunit protein complex. *Hum. Immun.* **15** pp. 416-26.

Monaco, J., and Nandi, D., (1995) The genetics of proteasomes and antigen processing. *Ann. Rev. Genet.* **29** pp. 729-54.

Monia, B., Ecker, D., Finley, D., and Crooke, S., (1990) A human ubiquitin extension protein functions in yeast. *J. Biol. Chem.* **265** pp. 19356-61.

Mori, H., Kondo, J., and Ihara, Y., (1987) Ubiquitin is a component of the paired helical filaments in Alzheimer's Disease. *Science* **235** pp. 1641-44.

Morrison, A., Miller, E., and Prakash, L., (1988) Domain structure and functional analysis of the carboxyl-terminal polyacidic sequence of the RAD6 protein of *Saccharomyces cerevisiae. Mol. Cell Biol.* **8** (3) pp.1179-85.

Mueller, R., Yasuda, H., Hatch, C., Bonner, W., and Bradbury, E., (1985) Identification of ubiquitinated histones 2A and 2B in *Physarum polycephalum. J. Biol. Chem.* **260** pp. 5147-53.

Muller, S., and Schwartz, L., (1995) Ubiquitin in homeostasis, development and disease. *BioEssays* **17** pp. 677-84.

Muralidhar, M., and Thomas, J., (1993) The *Drosophila bendless* gene encodes a neural protein related to ubiquitin-conjugating enzymes. *Neuron* **11** pp.253-266.

Murray, A., (1995) Cyclin ubiquitination: the destructive end of mitosis. *Cell* **81** pp. 149-52.

Neefjies, J., Momburg, F., and Hämmerling, G., (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* **261** pp. 769-71.

Nefsky, B., and Beach, D., (1996) PUB1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of CDC25. *EMBOJ.* **15** pp. 1301-12.

Nishizawa, M., Furuno, N., Okazaki, K., Tanaka, H., Ogawa, Y., and Bagata, N., (1993) Degradation of Mos by the N-terminal proline (Pro^2)-dependent ubiquitin pathway on fertilization of *Xenopus* eggs: possible significance of natural selection for Pro^2 in Mos. *EMBO J.* **12** pp. 4021-27.

Nuber, U., Schwarz, S., Kaiser, P., Schneider, R., and Scheffner, M., (1996) Cloning of human ubiquitin-conjugating enzymes *UbcH6* and *UbcH7* (E2-F1) and characterization of their interaction with E6-AP and RSP5. *J. Biol. Chem.* **271** pp. 2795-800.

O'Kane, C., and Gehring, G., (1987) Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **84** 9123-27.

Özkaynak, E., Finley, D., Solomon, M., and Varshavsky, A., (1982) The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J.* **6** pp. 1429-39.

Özkaynak, E., Finley, D., and Varshavsky, A., (1984) The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature* **312** pp. 663-66.

Palombella, V., Rando, O., Goldberg, A., and Maniatis, T., (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78** pp. 773-85.

Papa, F., and Hochstrasser, M., (1993) The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. *Nature* **366** pp. 313-19.

Parag, H., Dimitrovsky, D., Raboy, B., and Kulka, R., (1993) Selective ubiquitination of calmodulin by UBC4 and a putative ubiquitin protein ligase (E3) from *Saccharomyces cerevisiae. FEBS Lett.* **325** pp. 242-46.

Parkhurst, S., and Ish-Horowitz, D., (1991) Mis-regulating segmentation gene expression in *Drosophila*. *Development* **111** pp. 1121-35.

Peters, J., (1994) Proteasomes - protein degradation machines of the cell. *Trends Biochem. Sci.* **19** pp. 377-82.

Pickart, C., and Rose, I., (1985) Functional heterogeneity of ubiquitin carrier proteins. *J. Biol. Chem.* **260** pp. 1573-81.

Picologlou, S., Brown, N., and Liebman, S., (1990) Mutations in *RAD6*, a yeast gene encoding a ubiquitin-conjugating enzyme, stimulate retrotransposition. *Mol. Cell Biol.* **10** pp. 1017-22.

Pines, J., (1994) Ubiquitin with everything. *Nature* **371** pp. 742-43.

Pines, J., and Hunter, T., (1991) Human cyclin-A and cyclin-B1 are differentially located in the cell and undergo cell-cycle dependent nuclear transport. *J. Cell Biol.* **115** pp. 1-17.

Plon, S., Leppig, K., Do, H.-N., and Groudine, M., (1993) Cloning of the human homolog of the *CDC34* cell cycle gene by complementation in yeast. *Proc. Natl. Acad. Sci. USA* **90** pp. 10484-88.

Prendergast, J., Ptak, C., Arnason, T., and Ellison, M., (1995) Increased ubiquitin expression suppresses the cell cycle defect associated with the yeast ubiquitin conjugating enzyme, CDC34 (UBC3). Evidence for a noncovalent interaction between CDC34 and ubiquitin. *J. Biol. Chem.* **270** pp. 9347-52.

Prendergast, J., Ptak, C., Kornitzer, D., Steussy, N., Hodgins, R., Goebl, M., and Ellison, M., (1996) Identification of a positive regulator of the cell cycle ubiquitin-conjugating enzyme Cdc34 (Ubc3). *Mol. Cell Biol.* **16** pp. 677-84.

Ptak, C., Prendergast, J., Hodgins, R., Kay, C., Chau, V., and Ellison, M., (1994) Functional and physical characterization of the cell cycle ubiquitin-conjugating enzyme CDC34 (UBC3). Identification of a functional determinant within the tail that facilitates self-association. *J. Biol. Chem.* **269** pp. 26539-45.

Qin, S., Nakajima, B., Nomura. M., and Arfin, S., (1991) Cloning and characterisation of a *Saccharomyces cerevisiae* gene encoding a new member of the ubiquitin-conjugating protein family. *J. Biol. Chem.* **266** pp. 15549-54.

Raboy, B., Parag, H., and Kulka, R., (1986) Conjugation of [I-125] ubiquitin to cellular proteins in permeabilized mammalian cells- comparison of mitotic and interphase cells. *EMBO J.* **5** pp. 863-69.

Raleigh, E., Lech, K., and Brent, R., (1989) In: *Current Protocols in Molecular Biology* eds. Ausubel, F., *et al*. Publishing Associates and Wiley Interscience New York.

Realini, C., Rogers, S., and Rechsteiner, M., (1994) KEKE motifs - proposed roles in protein-protein association and presentation of peptides by MHC class-I receptors. *FEBS Lett.* **376** pp. 233-37.

Rechsteiner, M., (1990) PEST sequences are signals for rapid intracellular proteolysis. *Semin. Cell Biol.* **1** pp. 433-40.

Rechsteiner, M., (1991) Natural substrates of the ubiquitin proteolytic pathway. *Cell* **66** pp. 615-18.

Redman, K., (1994) The smaller protein formed as a ubiquitin fusion in *Drosophila* is processed from ubiquitin and found on the 60S ribosomal-subunit. *Insect Biochem. Mol. Biol.* **24** pp. 191-201.

Redman, K., and Rechsteiner, M., (1989) Identification of the long ubiqutin extension as ribosomal protein S27A. *Nature* **338** pp. 438-40.

Reiss, Y., and Hershko, A., (1990) Affinity purification of ubiquitin-protein ligase on immobilized protein substrates. *J. Biol. Chem.* **265** pp. 3685-90.

Reiss, Y., Kaim, D., and Hershko, A., (1988) Specificity of binding of NH_2-terminal residue of proteins to ubiquitin-protein ligase - use of amino-acid derivatives to characterize specific binding-sites. *J. Biol. Chem.* **263** pp. 2693-98.

Reynolds, P., Weber, P., and Prakash, L., (1985) *RAD6* gene of *Saccharomyces cerevisiae* encodes a protein containing a tract of 13 consecutive aspartates. *Proc. Natl. Acad. Sci. USA* **82** pp. 168-72.

Reynolds, P., Koken, M., Hoeijmakers, J., Prakash, S., and Prakash, L., (1990) The *rhp6*$^+$ gene of *Schizosaccharomyces pombe*: a structural and functional homolog of the *RAD6* gene from the distantly related yeast *Saccharomyces cerevisiae*. *EMBO J.* **9** pp. 1423- 30.

Roberts, D., (1986) Basic *Drosophila* care and techniques. In: *Drosophila a practical approach* ed. Roberts, D., IRL Press pp. 1-38.

Robertson, H., Preston, C., Phillis, R., Johnson-Schlitz, D., Benz, W., and Engels, W., (1988) A stable source of P element transposase in *Drosophila melanogaster*. *Genetics* **118** pp. 461-70.

Robin, C., Medveczky, K., Russell, R., and Oakeshott, J., (1996) Duplication and divergence of the genes of the alpha-esterase cluster of *Drosophila melanogaster*. *J. Mol. Evol.* in press.

Rock, K., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A., (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC classI molecules. *Cell* **78** pp. 761-71.

Rogers, S., Wells, R., and Rechsteiner, M., (1986) Amino-acid sequences common to rapidly degraded proteins - the PEST hypothesis. *Science* **234** pp. 364-68.

Rubin, D., and Finley, D., (1995) The proteasome: a protein degrading organelle? *Curr. Biol.* **5** pp. 854-58.

Rubin, D., and Spradling, A., (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218** pp. 348-53.

Sadis, S., Atienzya, C., and Finley, D., (1995) Synthetic signals for ubiquitin-dependent proteolysis. *Mol. Cell Biol.* **15** pp. 4086-94.

St Johnston, D., and Nüsslein-Volhard, C., (1992) The origin of pattern in the *Drosophila* embryo. *Cell* **68** pp. 201-19.

Samejima, I., and Yanagida, M., (1994) Bypassing anaphase by fission yeast *cut9* mutation - requirement of *cut9*$^+$ to initiate anaphase. *J. Cell. Biol.* **127** pp. 1655-70.

Sanger, F., Nicklen, S., and Coulson, A., (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74** pp. 5463-67.

Scheffner, M., Huibregtse, J., Vierstra, R., and Howley, P., (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75** pp. 495-505.

Scheffner, M., Nuber, U., and Huibregtse, J., (1995) Protein-ubiquitination involving an E1-E2-E3 enzymes ubiquitin thioester cascade. *Nature* **373** pp. 81-83.

Schmitz, A., Maintz, M., Kehle, T., and Herzog, V., (1995) *In vivo* iodination of a misfolded proinsulin reveals co-localized signals for BiP binding and for degradation in the E.R. *EMBOJ.* **14** pp. 1091-98.

Schwartz, L., (1991) The role of cell death genes during development. *BioEssays* **13** pp. 389-95.

Schwartz, L., Myer, A., Kasz, L., Engelstein, M., and Maier, C., (1990) Activation of polyubiquitin gene expression during developmentally programmed cell death. *Neuron* **5** pp. 411-19.

Schwob, E., Böhm, T., Mendenhall, M., and Nasmyth, K., (1994) The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1 to S transition in *S.cerevisiae*. *Cell* **79** pp. 223-44.

Seelig, A., Multhaup, G., Pesold-Hurt, B., Beyreuther, K., and Kloetzel, P.-M., (1993) *Drosophila* proteasome Dm25 subunit substitutes for the mouse MC3 subunit in hybrid proteasomes. The N-terminal domain is essential for subunit incorporation. *J. Biol. Chem.* **268** pp. 25561-67.

Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R., and Baumeister, W., (1995) Proteasome from *Thermoplasma acidophilum*: a threonine protease. *Science* **268** pp. 579-82.

Seufert, W., and Jentsch, S., (1990) Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* **9** (2) pp.543-50.

Seufert, W., McGrath, J., and Jentsch, S., (1990) *UBC1* encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. *EMBO J.* **9** pp.4535-41.

Seufert, W., Futcher, B., and Jentsch, S., (1995) Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature* **373** pp. 78-81.

Shanklin, J., Jabben, M., and Vierstra, R., (1987) Red light-induced formation of ubiquitin-phytochrome conjugates: identification of possible intermediates of phytochrome degradation. *Proc. Natl. Acad. Sci. USA* **84** pp. 359-61.

Silver, E., Gwozd, T., Ptak, C., Goebl, M., and Ellison, M., (1992) A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2): implications for the structure, function and evolution of the E2s. *EMBO J.* **11** (8) pp. 3091-98.

Sommer, T., and Jentsch, S., (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* **365** pp. 176-79.

Southern, E., (1975) Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. *J. Mol. Biol.* **98** pp. 503-17.

Stancovski, I., Gonen, H., Orian, A., Schwartz, A., and Ciechanover, A., (1995) Degradation of the protooncogene product c-Fos by the ubiquitin proteolytic system *in vivo* and *in vitro* - identification and characterization of the conjugating enzymes. *Mol. Cell. Biol.* **15** pp. 7106-16.

Stroumbakis, N., Li, Z., and Tolias, P., (1996) A homolog of human transcription factor NF-X1 encoded by the *Drosophila shuttle craft* gene is required in the embryonic central nervous system. *Mol. Cell. Biol.* **16** pp. 192-201.

Suenaga, T., Hirano, A., Llena, J., Ksiezak-Reding, H., Yen, S., and Dickson, D., (1990) Ubiquitin immunoreactivity in kuru plaques in Creutzfeldt-Jakob disease. *Ann. Neurol.* **28** pp. 174-77.

Sullivan, M., and Vierstra, R., (1991) Cloning of a 16kDa ubiquitin carrier protein from wheat and *Arabidopsis thaliana. J. Biol. Chem.* **267** pp. 15116-21.

Sullivan, M., and Vierstra, R., (1993) Formation of a stable adduct between ubiquitin and the *Arabidopsis* ubiquitin-conjugating enzyme ATUBC1+. *J. Biol. Chem.* **268** pp.8777-80.

Sung, P., Prakash, S., and Prakash, L., (1988) The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity. *Genes Dev.* **2** pp. 1476-85.

Sung, P., Prakash, S., and Prakash, L., (1990) Mutation of cysteine-88 in the *Saccharomyces cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. *Proc. Natl.Acad. Sci. USA* **87** pp. 2695-99.

Sung, P., Berleth, E., Pickart, C., Prakash, S., and Prakash, L., (1991) Yeast *RAD6* encoded ubiquitin conjugating enzyme mediates protein degradation dependent on the N-end recognizing E3 enzyme. *EMBO J.* **10** pp. 2187-93.

Surana, U., Armon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K., (1993) Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* **12** pp. 1969-78.

Swofford, D., Olsen, G., Waddell, P., and Hillis, D., (1996) Phylogenetic inference. In: *Molecular Systematics* ed. Hillis, D., and Morritz, C., Sinauer pp. 407-514.

Sussman, R., and Jacob, F., (1962) Sur un système de répression thermosensible chez le bacteriophage λ d'*Escherichia coli*. *CR Acad. Sci. (Paris)* **254** pp. 1517-19.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J., and Reed, J., (1995) Cloning and functional analysis of BAG-1 - a novel Bcl-2-binding protein with anti-cell death activity. *Cell* **80** pp. 279-84.

Tamura, T., Nagy, I., Lupas, A., Lottspeich, F., Cejka, Z., Schoofs, G., Tanaka, K., Demot, R., and Baumeister, W., (1995) The first characterization of a eubacterial proteasome - the 20S complex of Rhodococcus. *Current Biol.* **5** pp.

Tautz, D., and Pfeifle, C., (1989) A non-radioactive *in situ* hybridisation method for the localisation of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback. Chromosoma* **98** pp. 81-85.

Terlecky, S., and Dice, J., (1993) Polyprotein import and degradation by isolated lysosomes. *J. Biol. Chem.* **268** pp. 23490-95.

Thanos, D., and Maniatis, T., (1995) NF-kappa B - a lesson in family values. *Cell* **80** pp. 529-32.

Thomas, J., and Wyman, R., (1984) Mutations altering synaptic connectivity between identified neurons in *Drosophila*. *J. Neurosci.* **4** pp. 530-38.

Thompson, J., Higgins, D., and Gibson, T., (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc. Acids Res.* **22** pp. 4673-80.

Tower, J., Karpen, G., Craig, N., and Spradling, A., (1993) Preferential transposition of *Drosophila* P Elements to nearby chromosomal sites. *Genetics* **133** pp. 347-59.

Treier, M., Seufert, W., and Jentsch, S., (1992) *Drosophila UbcD1* encodes a highly conserved ubiquitin-conjugating enzyme involved in selective protein degradation. *EMBO J.* **11** pp. 367-72.

Treier, M., Staszewski, L., and Bohmann, D., (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the δ domain. *Cell* **78** pp. 787-98.

Tugendreich, S., Tomkiel, J., Earnshaw, W., and Hieter, P., (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell* **81** pp. 261-68.

Tyers, M., Tokina, G., Nash, R., and Futcher, B., (1992) The Cln3-Cdc28 kinase complex of *S.cerevisiae* is regulated by proteolysis and phophorylation. *EMBO J.* **11** pp. 1773-84.

Udvardy, A., (1993) Purification and characterization of a multiprotein component of the *Drosophila* 26S (1500kDa) proteolytic complex. *J. Biol. Chem.* **268** pp. 9055-62.

Vannocker, S., Deveraux, Q., Rechsteiner, M., and Vierstra, R., (1996) *Arabidopsis MBPI* gene encodes a conserved ubiquitin recognition component of the 26S proteasome. *Proc. Natl. Acad. Sci. USA* **93** pp. 856-60.

Varshavsky, A, The N-end rule. *Cell* **69** pp. 725-35.

Vierstra, R., (1994) Characterization of the ubiquitin conjugating system in plants. EMBO-RAPPAPORT Workshop.

Vijay-Kumar, S., Bugg, C., Wilkinson, K., and Cook, K., (1985) 3-Dimensional structure of ubiquitin at 2.8A resolution. *Proc. Natl. Acad. Sci. USA* **82** pp.3582-85.

Vijay-Kumar, S., Bugg, C., and Cook, W., (1987) Structure of ubiquitin refined at 1.8A resolution. *J. Molec. Biol.* **195** pp. 531-44.

Ward, C., Omwa, S., and Kopito, R. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83** pp. 121-27.

Watkins, J., Sung, P., Prakash, S., and Prakash, L., (1993) The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. *Genes Dev.* **7** pp. 250-61.

Webster, N., Jin, J., Green, S., Hollis, M., and Chambon, P., (1988) The yeast UAS_G is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 *trans*-activator. *Cell* **52** pp. 169-78.

Wefes, I., Mastrandrea, L., Haldeman, M., Koury, S., Tamburlin, J., Pickart, C., and Finley, D., (1995) Induction of ubiquitin-conjugating enzymes during terminal erythroid differentiation. *Proc. Natl. Acad. Sci. USA* **92** pp. 4982-86.

Weiler, K., and Wakimoto, B., (1995) Hereochromatin and gene expression in *Drosophila*. *Annu. Rev. Genetics* **29** pp. 577-605.

Wenzel, T., and Baumeister, W., (1995) Conformational constraints in protein degradation by the 20S proteasome. *Nature Structural Biol.* **2** pp. 199-203.

Wiborg, O., Pederson, M., Wind, A., Berglund, L., Marcker, K., and Vuust, J., (1985) The human ubiquitin multigene family - some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J.* **4** pp. 755-59.

Wiebel, F., and Kunau, W.-H., (1992) The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin conjugating enzymes. *Nature* **359** pp. 73-76.

Wieschaus, E., and Nüsslein -Volhard, C., (1986) Looking at embryos. In: *Drosophila a practical approach* ed. Roberts, D., IRL Press pp.199-227.

Wilkinson, K., (1994) Structure and processing of polymeric ubiquitin chains. EMBO-RAPPAPORT Workshop.

Wilkinson, K., Lee, K., Deshpande, S., Duerksen-Hughes, P., Boss, J., and Pohl, J., (1989) The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* **246** pp. 670-73.

Wilkinson, K., Tashayev, V., O'Connor, L., Larsen, C., Kasperek, E., and Pickart, C., (1995) Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochem*. **34** pp. 14535-46.

Wing, S., and Jain, P. (1995) Molecular cloning, expression and characterization of a ubiquitin conjugating enzyme (E2(17kB)) highly expressed in rat testis. *Biochem. J.* **305** pp. 125-32.

Wing, S., Dumas, F., and Banville, D., (1992) A rabbit reticulocyte ubiquitin carrier protein that supports ubiquitin-dependent proteolysis (E2(14K)) is homologous to the yeast DNA repair gene *RAD6*. *J. Biol. Chem.* **267** pp. 6495-501.

Wolf, S., Lottspeich, F., and Baumeister, W., (1993) Ubiquitin found in the archaebacterium *Thermoplasma acidophilum*. *FEBS Lett.* **326** pp. 42-44.

Worrall, D., and Twell, D., (1994) Pollen maturation - where ubiquitin is not required. *BioEssays* **16** pp. 873-75.

Yoon, H.-J., and Carbon, J., (1995) Genetic and biochemical interaction between an essential kinetochore protein, Cbf2p/Ndc10p, and the CDC34 ubiquitin-conjugating enzyme. *Mol. Cell Biol.* **15** pp. 4835-42.

Zachsenhaus, E., and Shenin, R., (1990) Molecular cloning, primary structure and expression of the human X linked *A1S9* gene cDNA which complements the t.s. A1S9 mouse L cell defect in DNA replication. *EMBO J.* **9** pp. 2923-29.

Zhang, N., (1992) Ph.D. thesis - Cloning and characterisation of the ubiquitin carboxyl terminal hydrolase gene in *Drosophila melanogaster*.

Zhang, N., Wilkinson, K., and Bownes, M., (1993) Cloning and analysis of a ubiquitin carboxyl-terminal hydrolase expressed during oogenesis in *Drosophila melanogaster*. *Dev. Biol.* **157** pp.214-23.

Zhang, P., and Spradling, A., (1993) Efficient and dispersed local P Element transposition from *Drosophila* females. *Genetics* **133** pp. 361-73.

Zhen, M., Heinlein, R., Jones, D., Jentsch, S., and Candido, E., (1993) The *ubc-2* gene of *Caenorhabditis elegans* encodes a ubiquitin conjugating enzyme involved in selective protein degradation. *Mol. Cell Biol.* **13** pp. 1371-77.

Zhou, L., Hashimi, H., Schwartz, L., and Nambu, J., (1995) Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol.* **5** pp. 784-90.